

# The role of P2Y receptors in bone; a microCT study

**Isabel R Orriss<sup>1</sup>, Timothy R Arnett<sup>1</sup>, Alison Gartland<sup>2</sup>, Bernard Robaye<sup>2</sup>, Jean-Marie Boeynaems<sup>3</sup>**

<sup>1</sup> Department of Cell & Developmental Biology, University College London, London, UK, [i.orriss@ucl.ac.uk](mailto:i.orriss@ucl.ac.uk)

<sup>2</sup> Mellanby Centre for Bone Research, Academic Unit of Bone Biology, University of Sheffield, Sheffield, UK.

<sup>3</sup> Insititute of Interdisciplinary Research (IRIBHM), Université Libre de Bruxelles, Belgium.

## Aims

All cells require energy to carry out the biochemical reactions necessary for survival; the free energy donor in most of these processes is adenosine triphosphate (ATP). ATP belongs to a large family of molecules essential in cellular function called nucleotides. Nucleotides are ubiquitous across all cell types and are involved in numerous intracellular biochemical processes and metabolic pathways. In addition, nucleotides can act on extracellular membrane receptors (P2 receptors) to influence biological processes in a wide range of tissues (1). The P2 receptors are subdivided into P2X receptors, which are ligand-gated ion channels, and the G-protein linked P2Y receptors. Seven P2X (P2X<sub>1,2,3,4,5,6,7</sub>) receptors and eight P2Y receptors (P2Y<sub>1,2,4,6,11,12,13,14</sub>) have been identified; each displays distinct tissue expression and pharmacology (1). Both osteoblasts, the bone-forming cells, and osteoclasts, the bone-resorbing cells, express multiple P2 receptor subtypes (2).

Accumulating evidence from *in vitro* studies suggests that P2Y receptors play an important role in regulating bone cell function. Activation of the P2Y<sub>1</sub> and P2Y<sub>6</sub> receptors by their ligands (adenosine diphosphate (ADP) and uridine diphosphate (UDP), respectively) stimulates osteoclast formation and resorption (3). In contrast, stimulation of the P2Y<sub>2</sub> receptor by ATP or uridine triphosphate (UTP) inhibits bone mineralization by osteoblasts (4).

Knockout mouse models have been generated for the P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors. Using the SkyScan 1172 we have performed the first *in vivo* study to determine the effects of receptor removal on bone structure.

## Methods

The long bones and spine were isolated from two-month old P2Y<sub>1</sub> (P2Y<sub>1</sub>R<sup>-/-</sup>), P2Y<sub>2</sub> (P2Y<sub>2</sub>R<sup>-/-</sup>) and P2Y<sub>6</sub> (P2Y<sub>6</sub>R<sup>-/-</sup>) knockout mice and their corresponding wildtypes (P2Y<sub>1</sub>R<sup>+/+</sup>, P2Y<sub>2</sub>R<sup>+/+</sup>, P2Y<sub>6</sub>R<sup>+/+</sup>) and fixed in formaldehyde for 24 hours. Bones were then washed in phosphate buffered saline and stored in 70% ethanol until scanning. MicroCT analysis of trabecular and cortical bone parameters was performed on the tibial and femoral metaphysis and the L3 lumbar vertebrae. In the tibia and femur, the appearance of the first cartilage bridge was used as a reference point, with an offset of 0.4mm and 2.5mm for trabecular and cortical bone, respectively. In the L3 vertebrae, the reference point was the first appearance of calcified tissue from the uncalcified end plate, with an offset of 70 slices. The microCT scanner was set at 50Kv and 200µA using a 0.5mm Al filter and a resolution of 4.7µm (8.3µm for the

spine). The images were reconstructed using the SkyScan NRecon program and analysed using SkyScan CTAn software.

## Results

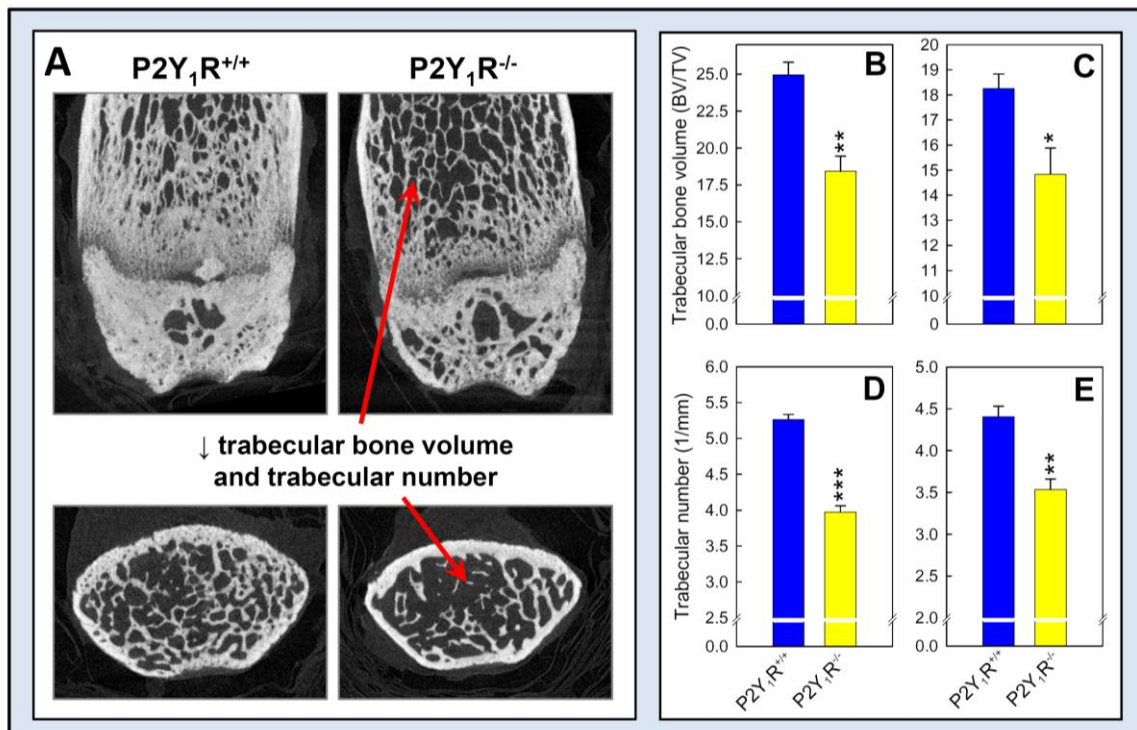
All three of the knockout models displayed no overt changes in phenotype. Weight, lean tissue and fat content were unchanged.

### *P2Y<sub>1</sub>R<sup>-/-</sup> mice*

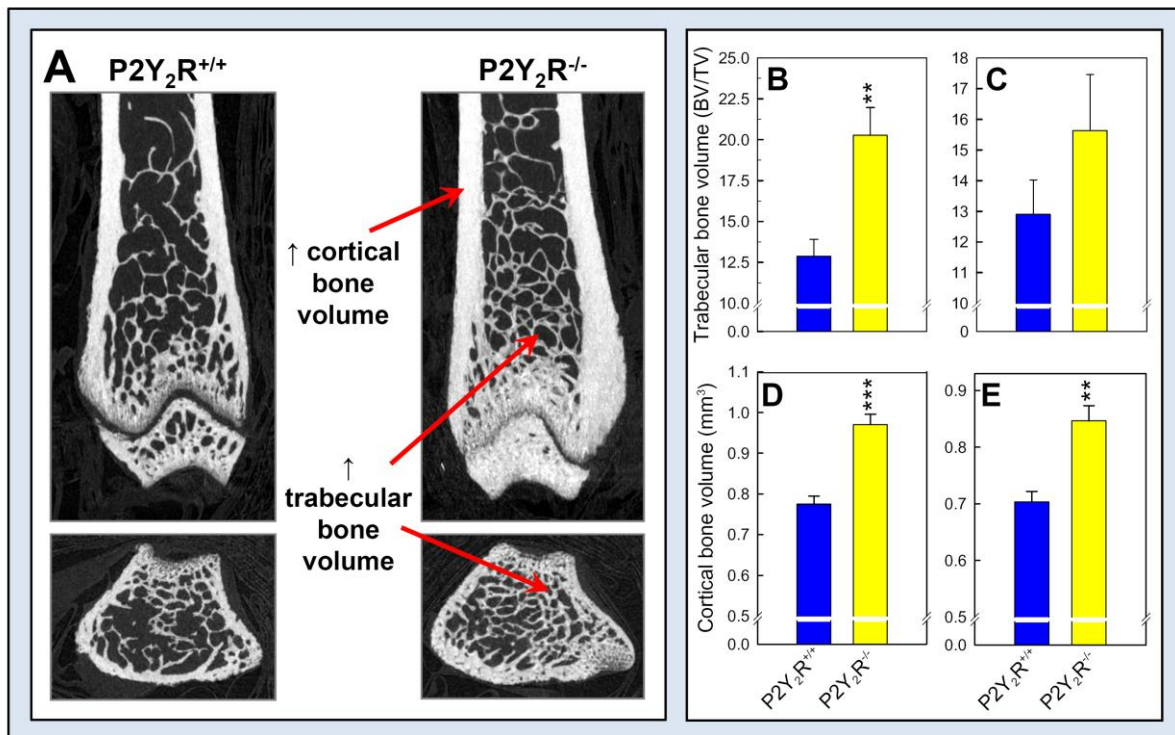
Detailed analysis of trabecular bone (**Fig. 1A**) showed significant decreases in the trabecular bone volume (BV/TV) of the P2Y<sub>1</sub>R<sup>-/-</sup> femur (35%,  $p < 0.01$ ) (**Fig. 1B**) and tibia (23%,  $p < 0.05$ ) (**Fig. 1C**). Quantification of trabecular number showed a reduction of 32% in the femur, ( $p < 0.01$ ) (**Fig. 1D**) and 25% in the tibia ( $p < 0.01$ ) (**Fig. 1E**); however, trabecular thickness was unchanged. The structural model index (SMI) was increased in both the femur and tibia indicating that the trabeculae were more rod-like in the P2Y<sub>1</sub>R<sup>-/-</sup> mice. No changes in cortical bone volume were observed.

### *P2Y<sub>2</sub>R<sup>-/-</sup> mice*

In the P2Y<sub>2</sub>R<sup>-/-</sup> mice significant changes in bone architecture were observed (**Fig. 2A**); BV/TV was increased by 43% ( $p < 0.01$ ) in the femur (**Fig. 2B**) and 21% in the tibia (**Fig. 2C**). Increases in femoral trabecular thickness (17%,  $p < 0.01$ ) and trabecular number (33%,  $p < 0.05$ ) were also observed. A decreased SMI indicated that the trabeculae were more plate like in the femora of P2Y<sub>2</sub>R<sup>-/-</sup>, whilst a reduced trabecular pattern factor suggests the trabeculae were less connected in knockout animals. Cortical bone volume was increased 25% ( $p < 0.001$ ) and 20% ( $p < 0.01$ ) in the femur (**Fig. 2D**) and tibia (**Fig. 2E**), respectively.



**Figure 1.** (A) P2Y<sub>1</sub>R<sup>-/-</sup> mice have reduced trabecular bone in the long bones. BV/TV was decreased 35% and 23% in the femur (B) and tibia (C), respectively. Trabecular number was reduced 32% in the femur (D) and 25% in the tibia (E).



**Figure 2.** (A) P2Y<sub>2</sub>R<sup>-/-</sup> mice have increased trabecular and cortical bone. BV/TV was increased 43% and 21% in the femur (B) and tibia (C), respectively. Cortical bone volume was increased 25% in the femur (D) and 20% in the tibia (E).

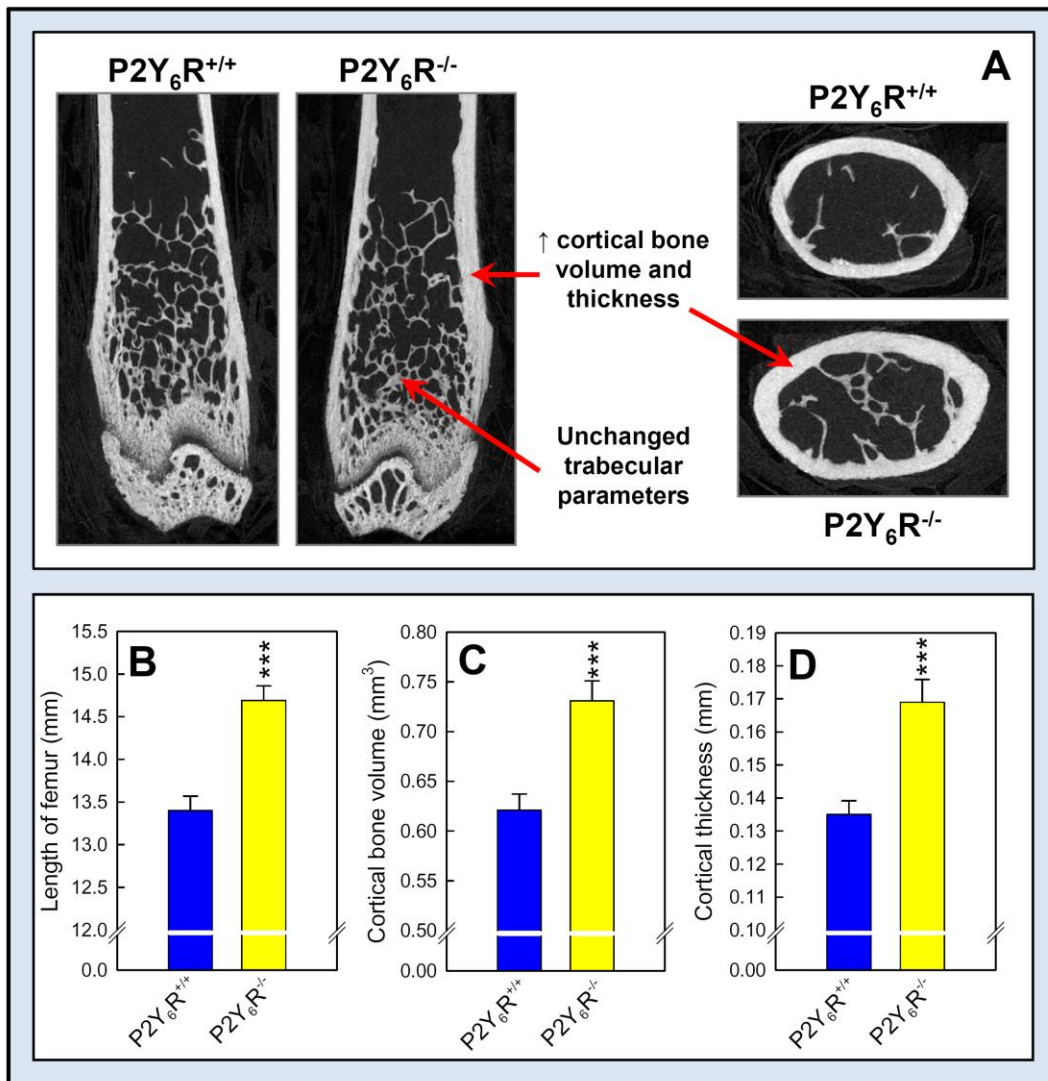
### P2Y<sub>6</sub>R<sup>-/-</sup> mice

In depth analysis of the P2Y<sub>6</sub>R<sup>-/-</sup> mice demonstrated significant changes in the cortical bone (Fig. 3A). The length of the long bones was increased up to 10% in the P2Y<sub>6</sub>R<sup>-/-</sup> mice (Fig. 3B). Increased femoral (18%, p<0.001) (Fig. 3C) and tibial (14%, p<0.01) cortical bone volume was also observed. Cortical thickness was increased up to 25% (p<0.001) (Fig. 3D). Long bone BV/TV and trabecular number were unaffected, however, trabecular thickness was increased 12% (p<0.001). Analysis of the L3 lumbar vertebrae demonstrated a 39% (p<0.01) increase in cortical bone volume whilst trabecular parameters were unaffected.

### Conclusions

These microCT data are the first *in vivo* evidence to show a role for the P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors in the regulation of bone cell function. In the case of the P2Y<sub>1</sub><sup>-/-</sup> mice, the differences in the trabecular architecture were somewhat unexpected given that P2Y<sub>1</sub> receptor activation stimulates bone resorption *in vitro*. Consequently, the effects of receptor deletion may predominantly affect osteoblast function. The increased trabecular bone parameters in the P2Y<sub>2</sub>R<sup>-/-</sup> mice are consistent with the *in vitro* effects on mineralisation; thus, deletion of the P2Y<sub>2</sub> receptor could potentially limit the negative actions of extracellular nucleotides on bone. Activation of the P2Y<sub>6</sub> receptor stimulates osteoclast activity and cells derived from these animals display impaired resorption. Consistent with osteoclast-mediated effects, microCT analysis showed increased cortical bone in the knockout animals.

These microCT data provide further evidence for the important role of purinergic receptors in modulating bone remodelling *in vivo*.



**Figure 3.** (A) P2Y<sub>6</sub>R<sup>-/-</sup> display increased cortical bone. (B) Femoral length was increased 10%. In the femur, cortical bone volume was increased 18% (C) and cortical thickness increased 25% (D).

## References

1. Burnstock G.. Purine and pyrimidine receptors. *Cell Mol Life Sci* 2007;64:1471-1483.
2. Orriss IR, Burnstock G, Arnett TR. Expression of multiple P2 receptor subtypes by osteoblasts and osteoclasts. *Bone* 2009;44:S304-S304.
3. Hoebertz A, Meghji S, Burnstock G, Arnett TR. Extracellular ADP is a powerful osteolytic agent: evidence for signaling through the P2Y1 receptor on bone cells. *FASEB J* 2001;15:1139-1148.
4. Orriss IR, Utting JC, Brandao-Burch A, Colston K, Grubb BR, Burnstock G, Arnett TR. Extracellular nucleotides block bone mineralization in vitro: evidence for dual inhibitory mechanisms involving both P2Y2 receptors and pyrophosphate. *Endocrinology* 2007;148:4208-4216.