

# Investigating Zoledronate Treatment as a Preventative Strategy for Paget's Disease of Bone

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## Aims

Paget's disease of bone (PDB) is a common bone disease affecting approximately 3% of the population over the age of 55. It is characterized by focally increased bone turnover caused by hyperactive osteoclasts, the bone resorbing cells. PDB leads to bone pain, bone deformation and associated complications such as osteoarthritis, deafness and rarely osteosarcoma. PDB is often not diagnosed until skeletal complications have already occurred. Treatment that inhibits the osteoclasts, using drugs called bisphosphonates, is very successful at reducing bone pain, but does not lead to repair of damaged bones and joints.

PDB is a disease with a strong genetic component, and the most common mutations leading to PDB are mutations in the SQSTM1 gene. This opens up the possibility of screening family members of current patients for the presence of these mutations, and treating carriers of the mutations with bisphosphonates before complication arise, thereby preventing the skeletal damage, pain and disability caused by the disease.

We have recently developed a mouse model of PDB, which carries the most common PDB-associated mutation in the SQSTM1 gene, the P394L mutation<sup>1</sup>. With ageing, these mice develop lesions comparable to those observed in human patients (see Fig. 1). The aim of this study was to investigate if treatment of young adult P394L mice, that have no PDB lesions yet, with the bisphosphonate zoledronate, prevents the development of PDB.

## Method

Mice homozygous for the P394L mutation were injected intraperitoneally with zoledronate at a dose of 85 ng/g body weight or vehicle control (PBS) every 2 months from the age of 4 months (N=10). The dose used is equivalent to the dose most commonly used in humans. At the age of 12 months, the mice were humanely killed, the hind limbs harvested, fixed overnight in 4% phosphate buffered formaldehyde, washed in PBS and stored in 70% ethanol. The mice were labeled with intra-peritoneal injections of calcein green (Sigma, UK) 5 days and 2 days prior to culling to enable histological analysis of bone formation.

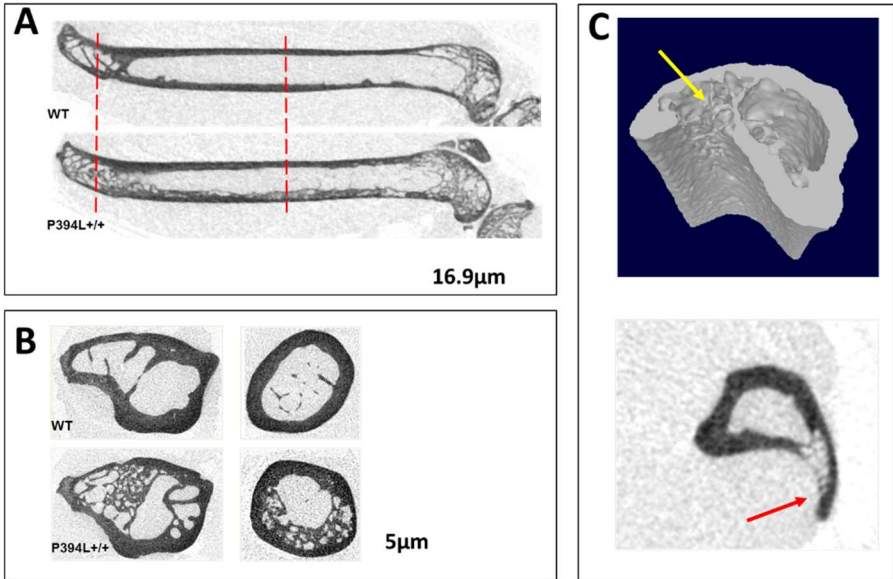


Figure 1: Development of Pagetic lesions in mice homozygous for the P394L mutation. Panel A shows a comparison of a normal WT femur (WT) and the femur of a P394L mutant mouse at 12 months of age. Note the abnormal spongy appearance of the cortex of the mutant mouse. Panel B shows transaxial slices at higher resolution from the same bone, taken at the level indicated by the dotted red line in panel A. Note the abnormal structure of the bone. Panel C shows a 3D image created using CTVox of a Pagetic lesion in the tibia of a mutant mouse, indicated by the yellow arrow. The lower image shows the typical appearance of this lesion in Dataviewer.

Both hind limbs of each mouse were scanned using a Skyscan 1172 μCT scanner, fitted with a robotic sample changer. The hind legs were scanned inside 1ml syringes in 70% ethanol. Initial scans were performed of the entire hind limbs at a resolution of 9 μm, X-ray source at 50kV and 200 μA and a 0.5 mm Al filter using the oversize scanning option. The rotation step size was 0.5°, and the camera was set to 2x2 binning. The resulting scans were reconstructed using NRecon, and analysed for the presence of pagetic-like lesions using the Dataviewer and CTVox programs. Additional scans used to analyse bone micro-architecture were performed at the distal femur at a resolution of 4.5 μm, without camera binning, and a rotation step size of 0.3°. Scans were reconstructed using NRecon and analysed using CTAn.

Finally, to analyse tissue mineralization, samples were equilibrated overnight in water, and the distal femur scanned inside drinking straws. The scanner settings were: resolution 4.5 μm, X-ray source at 50 kV and 200 μA, 0.5 mm Al filter, camera binning 2x2, rotation step size 0.3°, averaging at 3. Hydroxy-apatite standards (Skyscan, Belgium), were scanned using identical settings to calibrate mineral density.

For further histological analysis, the bones were embedded in methylmethacrylate, and sections cut at 5  $\mu\text{m}$ . The sections were stained with von Kossa/van Giesson to visualize mineralized and unmineralised (osteoid) bone respectively. Goldner's trichrome, stain was used for general imaging of matrix and cells. Tartrate resistant acid phosphatase (TRAP) staining was used to identify osteoclasts, and a calcein blue counterstain was used for imaging the calcein green bone labels. Sections were imaged on a Zeiss Axiolmager motorized microscope.

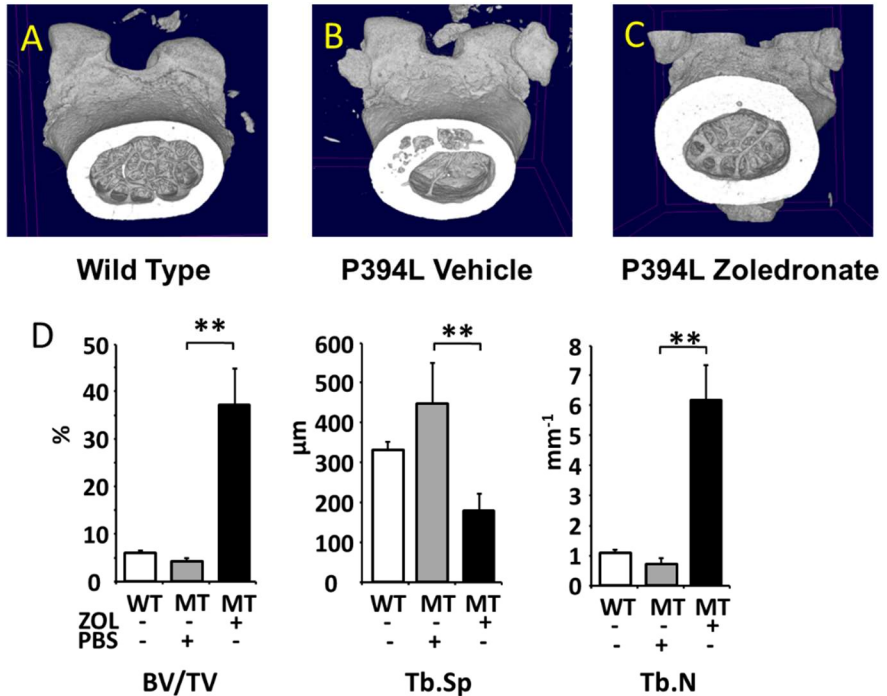


Figure 2: Zoledronate treatment prevents the development of Pagetic lesions in P394L mutant mice.

Bones were scanned at a resolution of 4.5  $\mu\text{m}$ , and visualized using CTVox. Panel A shows the distal femur of a wild type mice, and pane B| a typical femur cortical lesion in a P394L mutant mouse. Panel C shows the absence of lesions in the zoledronate treated mice. However, the cortex is abnormally thick. Panel D shows the results of the trabecular bone analysis, performed using CTAn. Zoledronate treatment resulted in significant increases in trabecular bone volme (BV/TV) and trabecular number (Tb.N) and a decrease in trabecular separation (Tb.Sp). \*\*:  $p < 0.01$ .

**Results**

At 12 months of age, all ten mutant mice treated with vehicle developed pagetic lesions in the femurs and tibias. Figure 2 shows a typical lytic lesion in the femoral cortex of a mutant mouse. None of the wild type or zoledronate treated mice developed lesions. However, the

bones of the zoledronate treated mice exhibited an abnormally high trabecular bone volume and substantially increased cortical bone thickness (Figure 2C). Analysis of the trabecular bone showed a dramatic increase of bone volume in the zoledronate treated mice, compared to both the vehicle treated and wild type mice (Figure 2D). This was accompanied by an increase in trabecular number and a reduction in trabecular separation.

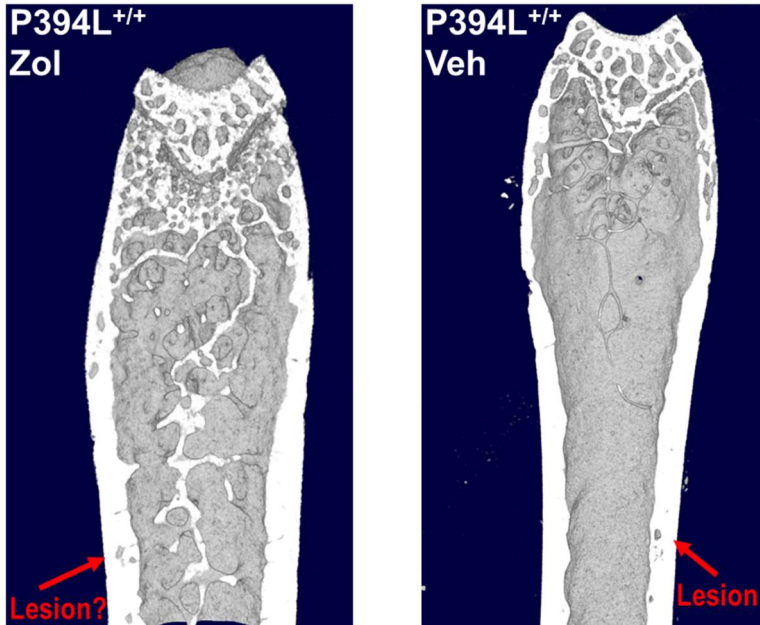


Figure 3: Abnormal cortical pores in zoledronate treated mice. Femurs were scanned at 4.5  $\mu\text{m}$  resolution and visualized using CTVox. Note the numerous pores in the thickened cortical bone of the zoledronate treated mice.

Although typical pagetic lesions were not observed in the zoledronate treated mice, we did observe frequent irregularly sized pores in the cortical bone of zoledronate treated mice. Pagetic lesions are characterized by the presence of large numbers of very large osteoclasts and increased bone formation. To investigate whether the observed pores were possibly “mini” pagetic lesions we analysed sections of these samples by histology.

TRAP staining revealed many, large osteoclast in a pagetic lesions in a vehicle mouse femur (indicated by the red arrows in figure 4) and extensive calcein double labels (indicated by yellow arrow in figure 4). The pores observed by  $\mu\text{CT}$  in the zoledronate treated bone (indicated by green arrow in detail inset in figure 4) showed a complete absence of osteoclasts. These structures were also devoid of calcein double labels (see detail inset top right figure 4). These findings suggest that the cortical pores in the zoledronate treated mice are not pagetic lesions, and that bone turnover is significantly suppressed in these animals.

Low bone turnover tends to result in hyper mineralization of the bone matrix. During sectioning for histology, we observed that the zoledronate treated samples were extremely brittle, and caused substantial damage to the microtome knives, further suggesting hyper mineralization.

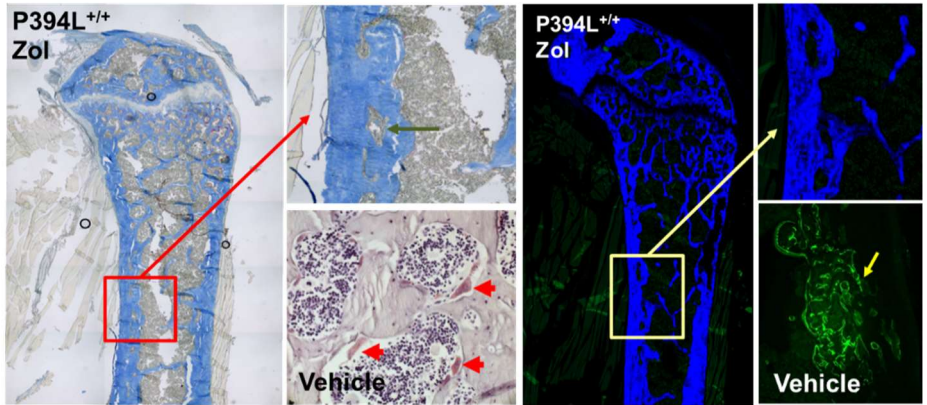


Figure 4: Cortical porosities in zoledronate treated mice do not resemble pagetic lesions. Note the absence of TRAP stained osteoclasts and calcein green double labels in the detail inset images. Sections from pagetic lesions in vehicle control mice showed numerous red stained osteoclasts (red arrows) and extensive calcein double labels (yellow arrow).

To investigate whether the zoledronate treated bones were indeed hyper mineralised, we performed additional scans of the distal femurs, with increased averaging and camera binning, to analyse the tissue mineralization in these bones. Figure 5A and B show the difference in image noise between the standard acquisition for morphology (A) and the density scan settings (B). A visual comparison of femurs from vehicle and zoledronate treated mice, already shows an increase in highly attenuating voxels in the zoledronate treated mice, as indicated by the increase in blue voxels in figure 5D versus predominantly green voxels in figure 5C. To quantify these images, we performed a calibration using 2 standards with  $0.25 \text{ g/cm}^3$  and  $0.75 \text{ g/cm}^3$  of hydroxyapatite respectively. Next, datasets consisting of 300 slices at the mid shaft of the femur were thresholded to identify the bone, and the resulting binary was eroded (3D space) with a sphere with a radius of 2 to remove voxels affected by partial voxel effects. This binary was then used as a mask to measure the mineral density in mineralized tissue only (the tissue mineral density or TMD). This analysis showed a TMD of  $1.42 \pm 0.016 \text{ g/cm}^3$  for the vehicle treated mice, and  $1.48 \pm 0.14 \text{ g/cm}^3$  for the zoledronate treated mice ( $p < 0.001$ ), clearly demonstrating increased bone mineralization in the zoledronate group.

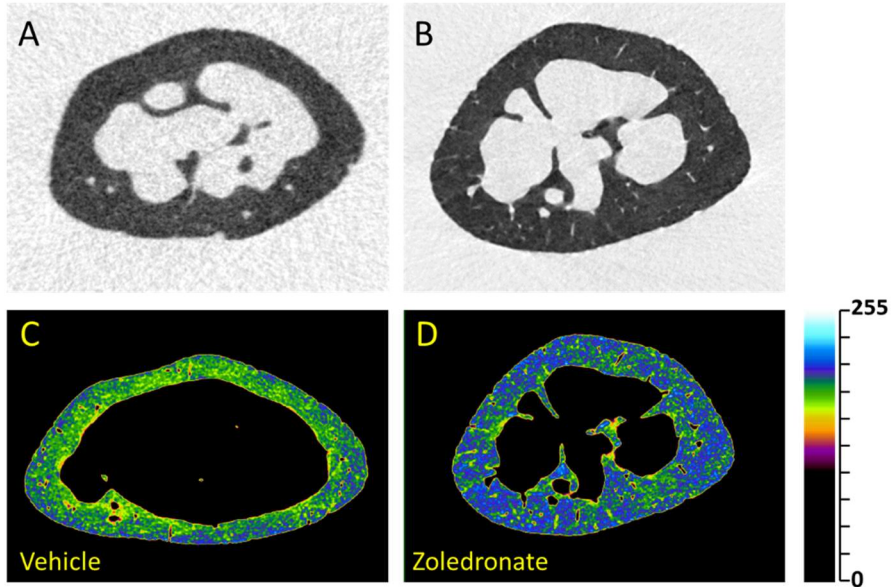


Figure 5: Analysis of tissue mineralization.

A: transaxial image of femur obtained using standard scan settings. B: transaxial image obtained using density scan settings. Note the substantial reduction in image noise in B compared to A. C and D are colour coded images of vehicle and zoledronate treated femurs respectively. The lookuo table used is shown in the scale next to D. Note the increase in blue pixels in D, indicating higher X-ray attenuation.

## Conclusion

Treatment of the P394L mutant mice with zoledronate completely prevented the development of pagetic lesions. However, the treatment also resulted in a very strong suppression of normal bone turnover, and resulted in hyper-mineralised, brittle bone. The increased mineralization may make the bone more prone to fracture.

Our findings may have implications for the long term treatment of individuals carrying Paget's-associated mutations of the SQSTM1 gene. A careful evaluation of the benefits and possible drawbacks of long term zoledronate treatment of young, still unaffected SQSTM1 mutation carriers should be made before this treatment is started.

## References:

1. Daroszewska A, van 't Hof RJ, Rojas JA, Layfield R, Landao-Basonga E, Rose L, Rose K, Ralston SH. A point mutation in the ubiquitin-associated domain of SQSTM1 is sufficient to cause a Paget's disease-like disorder in mice. *Hum Mol Genet* 20:2734-44, 2011