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Title:

Subchondral osteopenia and accelerated bone remodelling post-ovariectomy – a possible mechanism for subchondral microfractures in the aetiology of spontaneous osteonecrosis of the knee?

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Abstract:

Osteopenia and subchondral microfractures are implicated in the aetiology of spontaneous osteonecrosis of the knee. The ovine tibia shows significant alterations of the trabecular architecture within the subchondral bone of the medial tibial plateau post-ovariectomy, including reduced trabecular bone volume fraction. We hypothesise that accelerated subchondral bone resorption may also play a role in increasing microfracture risk at this site. 23 sheep were examined in this study; 10 of the sheep underwent ovariectomy (OVX), while the remainder (n=13) were kept as controls (CON). Five fluorochrome dyes were administered intravenously at 12 week intervals via the jugular vein to both groups, to label sites of bone turnover. These animals were then sacrificed at 12 months post-operatively. Bone turnover was significantly increased in the OVX group in both trabecular bone (2.024 vs. 1.047, $p = 0.05$) and within the subchondral bone plate (4.68 vs. 0.69 # / mm²; $p < 0.001$). In addition to the classically-described turnover visible along trabecular surfaces, we also found visual evidence of intra-trabecular osteonal remodelling. In conclusion, this study shows significant alterations in bone turnover in both trabecular bone and within the subchondral bone plate at one-year post-ovariectomy. Remodelling of trabecular bone was due to both classically described hemi-osteonal and intra-trabecular osteonal remodelling. The presence of both localised osteopenia and accelerated bone remodelling within the medial tibial plateau provide a possible mechanism for subchondral microfractures in the aetiology of spontaneous osteonecrosis of the knee. Further utilisation of the ovariectomised ewe may be useful for further study in this field.

Keywords:

Osteoporosis, Osteonecrosis, Osteonecrosis, remodelling, bone turnover, ovariectomy

Introduction:

The skeleton is constantly undergoing adaptation in response to mechanical stimuli (Wolff, 1870). Bone turnover has been investigated in the past with regard to its influence on fracture risk (Riggs and Melton, 2002); it has been demonstrated that accelerated bone resorption is associated with an increased incidence of osteoporotic fractures, independent of BMD (Meier et al., 2005). In skeletally mature animals, new bone may be deposited as a result of either modelling or remodelling. Modelling can be described as either bone formation occurring in the absence of prior resorption, or bone resorption without immediate bone formation occurring at the same surface. In contrast to bone modelling, remodelling involves the processes of bone resorption and formation occurring in succession. Both processes, however, involve the deposition and subsequent mineralisation of osteoid by osteoblasts (Buckwalter, 1987, Standring et al., 2005). The majority of studies that investigate osteoporotic bone quality tend to focus on trabecular bone tissue. This is because of the belief that the rate of bone turnover is higher in areas of trabecular bone; thus most of the deterioration in bone quantity and quality, including microarchitecture, will be found in these areas (Bilezikian et al., 2004). Neither of these suppositions is necessarily true. Parfitt noted that *'it has often been asserted, without qualification, that cancellous bone has higher turnover than cortical bone. (Parfitt, 2002)'* He went on to comment that there are circumstances in which this is indeed true, but there are also circumstances in which it is not.

Ovariectomized (OVX) sheep are accepted as useful models of accelerated bone turnover for a variety of metabolic bone disorders, including bone mineral density loss and alterations in trabecular bone architecture associated with oestrogen deficiency (Brennan et al., 2009, Kennedy et al., 2009a, Johnson et al., 2002, Newton et al., 2004, Thorndike and Turner, 1998, Holland et al., 2011). The ovine stifle joint may be considered to be a 1:3 scale model

of the human knee joint, with only minor morphological exceptions and the ovine bone remodelling cycle is comparable to that of humans, being of approximately 3 months duration (Lee et al., 2002, Osterhoff et al., 2011). We have previously confirmed that significant alterations of the trabecular architecture are present within the subchondral bone of the ovine tibial plateau at 12 months post-ovariectomy, specifically a diminished trabecular bone volume fraction, trabecular narrowing and reduced connectivity density (Holland et al., 2011); these changes are consistent with those elsewhere in the ovine skeleton (Jiang et al., 2005, Turner et al., 1995). This study attempts to now examine the link between ovariectomy and accelerated bone turnover at this site. Accelerated bone resorption is associated with an increased incidence of osteoporotic fractures, independent of Bone Mineral Density (BMD) (Hernandez, 2008, Meier et al., 2005), and microfractures within osteoporotic subchondral bone are thought to be a possible aetiological mechanism for spontaneous osteonecrosis of the knee (SPONK) (Lotke and Ecker, 1988, Yamamoto and Bullough, 2000).

Spontaneous osteonecrosis of the knee typically occurs over the age of 55 and is a recognised cause of knee pain; women tend to have a higher incidence than men (Akamatsu et al., 2012, Lotke et al., 1977). It usually affects one condyle of the knee, either femoral or tibial, and often leads to arthritic changes (Mears et al., 2009). MRI is used to detect these lesions within the medial femoral condyle or tibial plateau, but scintigraphy is considered superior (Satku et al., 2003). The initiating cause is thought to be traumatic, due to an insufficiency stress fracture of the medial femoral condyle or the medial tibial plateau. Subchondral insufficiency fractures secondary to osteopenia are also well documented within the subchondral bone of the femoral head; rapid progression to osteoarthritis or joint destruction in both the hip and knee have been reported following diagnosis (Satku et al., 2003, Yamamoto and Bullough, 2000).

Methods:

ANIMALS AND STUDY DESIGN

Twenty-two skeletally mature ewes were included in this study; approval was obtained by the ethics committee in the School of Veterinary Science in University College Dublin and an animal license, number B100/2443, was granted by the Department of Health under the Cruelty to Animals Act, 1876. The precise age of the animals was not known but the range was between 5-9 years. Animals were randomly allocated to ovariectomized or control groups; ovariectomy was performed on 10 of the sheep (OVX), while the remainder (n = 12) were kept as controls (CON). Fluorochrome dyes were administered intravenously via the jugular vein to both groups to label sites of bone turnover (Table 1). These were given at surgery and then at 12 weeks intervals; doses were individually calculated according to body mass (Kennedy et al., 2009b). The sheep were kept at pasture for 12 months and then sacrificed. Bones were immediately harvested and stored at -20°C.

Weeks post ovariectomy	Fluorochrome administered	Supplier	Dosage (mg/kg)
0	Oxytetracycline	Pfizer	50
12	Alizarin Complexone	Sigma-Aldrich	25
24	Calcein	Sigma-Aldrich	10
36	Xylenol Orange	Sigma-Aldrich	90
48	Calcein Blue	Sigma-Aldrich	30
52	Sacrifice		

Table 1. Schedule and doses for intravenous fluorochrome administration

SPECIMEN PREPARATION

Removal of the plateau from the intact ovine tibia was initially performed by use of a Struers Minitom Diamond Saw (Holland et al., 2011). The intact tibia was placed in the specimen holder of the Diamond Saw, and the proximal 1.5 – 2cm of the tibia removed. Following this, further cuts were made using the diamond saw to remove a 7 x 5mm osteochondral specimen (approx. 15mm deep) from the anterior aspect of the medial plateau. Three-dimensional analyses were performed of these specimens by MicroCT in order to examine the microarchitecture of the subchondral trabeculae and subchondral plate as previously described (μ CT40; Scanco Medical, Basserdorf, Switzerland) (Holland et al., 2011). For analysis of the subchondral trabecular bone, a volume of interest (VOI) was defined within the sample (Holland et al., 2011). A 3-dimensional image was then reconstructed by the Scanco software, with automated analysis of standard morphological parameters, including bone volume fraction (BV/TV), trabecular number, trabecular thickness, trabecular separation, connectivity density and hydroxyapatite concentration (Parfitt et al., 1987). Specimens were then cleaned with a water jet and dehydrated in 70% ethanol for 5 days. Further dehydration was performed over a period of 12 hours in graded ethanol (-20psi vacuum at room temperature). Following this, specimens were infiltrated in Methyl Methacrylate solution (MMA), prior to final embedding in Polymerised Methyl Methacrylate (PMMA) (O'Brien et al., 2000). Slides were prepared for microscopy as follows: histological sections with a thickness of approximately 120 – 150 μ m were taken from PMMA-embedded osteochondral specimen using a diamond saw (Struers, Accutom 50, Ballerup, Denmark). Sections were reduced to approximately 100 μ m, by grinding in aqueous solution, rinsed in distilled water, briefly dipped in Xylene and finally mounted on glass slides (using glass cover slips) with DPX.

MICROSCOPIC ANALYSIS

Each slide was examined using bright field microscopy (Nikon Eclipse 90i). Initial basic measurements were performed using a digital image analysis system (NIS Elements BR 3.0, Nikon). Each slide was then examined using a combination of ultra-violet (UV) ($\lambda=365\text{nm}$), blue ($\lambda=470\text{nm}$) and green ($\lambda=546\text{nm}$) epifluorescence microscopy at X10 magnification.

Within the trabecular bone, an area of interest measuring 4 x 7.5cm was analysed from each specimen, lying at a depth of 2.5cm from the calcified surface (Figure 1). The total area and area of bone was calculated for each area of interest. Each slide was then examined using a combination of ultra-violet (UV) ($\lambda=365\text{nm}$), blue ($\lambda=470\text{nm}$) and green ($\lambda=546\text{nm}$) epifluorescence microscopy at X10 magnification. Bone turnover was assessed by measuring both the number and length of sites with fluorochrome-labelled bone along the trabecular surfaces per measured bone area. These histomorphometric parameters are derived from the American Society for Bone and Mineral Research (ASBMR) nomenclature (Parfitt et al., 1987, Schorlemmer et al., 2005) . The bone turnover within the subchondral bone plate was quantified by calculating the numerical density of labelled secondary osteons per unit width of the subchondral specimen ($\#/mm$), and per unit area ($\#/mm^2$).

STATISTICAL ANALYSIS

PASW Statistics 18 (IBM® SPSS®) was used for data analysis. Q-Q plots were performed of all data sets to check for normal distribution. A t-test was performed if the appropriate criteria were met; otherwise a Mann-Whitney-U test was performed. Differences were considered significant for values of $p<0.05$

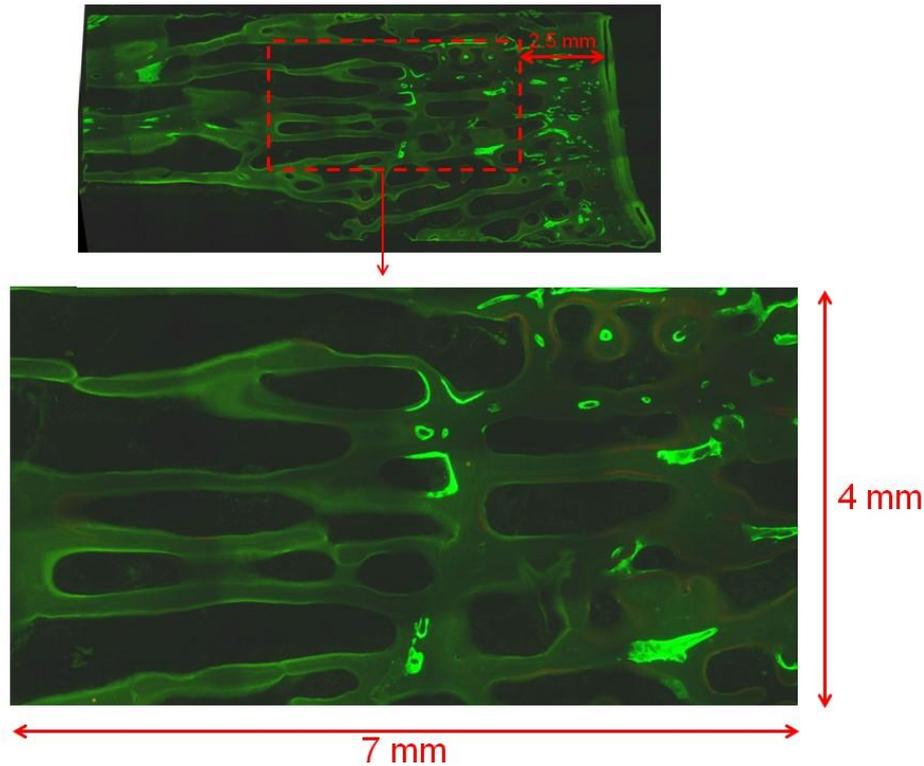


Figure 1. Defining the Area of Interest within the subchondral trabecular bone for epifluorescence microscopy

Results:

The specimen from one sheep was excluded from analysis of trabecular turnover, as it was damaged during removal from the glass vial following embedding in PMMA. The subchondral bone was intact and so still included in the analysis of subchondral turnover. This sheep was one of the control animals.

As previously reported by the authors, three-dimensional analyses showed that trabecular thickness, bone volume fraction and connectivity density are all significantly reduced in the OVX group as compared to controls, but that the material density, as measured by hydroxyapatite concentration, is unaffected (Holland et al., 2011). Bone turnover along the trabecular surfaces was elevated following ovariectomy; the number of sites of labelled bone

present per unit area of bone (BV) was higher in OVX sheep as compared to controls (2.024 vs. 1.047, $p = 0.05$, Mann-Whitney U test; Figure 2). When the total length of the sites of labelled bone per area was calculated, the OVX group again had a longer length of labelled bone present along trabecular surfaces per unit area of bone (0.913 vs. 0.508, $p = 0.067$, Mann-Whitney U test; Figure 2).

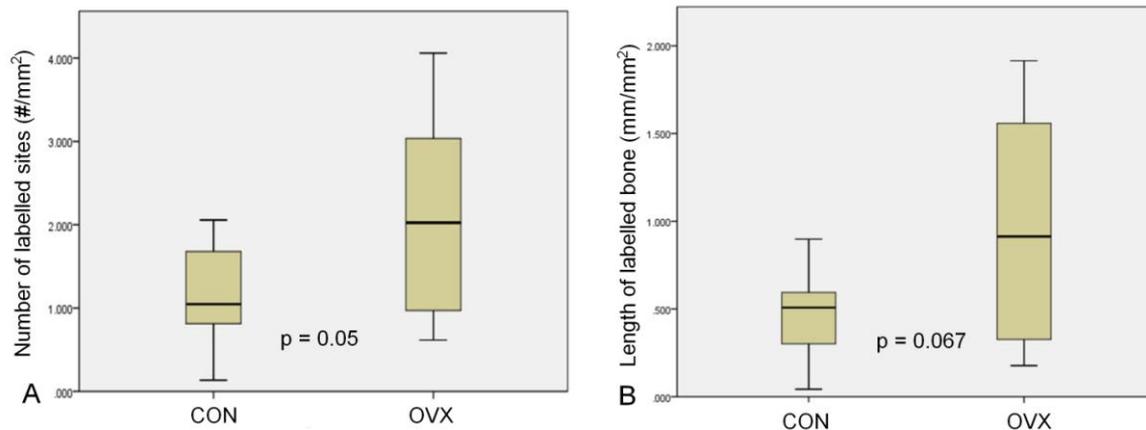


Figure 2. Comparison of trabecular bone turnover between the two groups

In addition to the labelled bone sites visualised along the trabecular surfaces, there were also areas of labelled bone within the trabeculae. The majority of these sites had the appearance of being cylindrical lamellae of bone, seen in a variety of planes, arranged around a central vessel - characteristic of secondary osteons formed by osteonal remodelling (Figure 3).

When the number of these sites of intra-trabecular labelled lamellar bone was quantified, the number of sites present per unit area (mm^2) was much higher in the OVX Group, but this failed to reach significance (0.285 vs. 0.13, $p = 0.084$, Mann-Whitney U test).

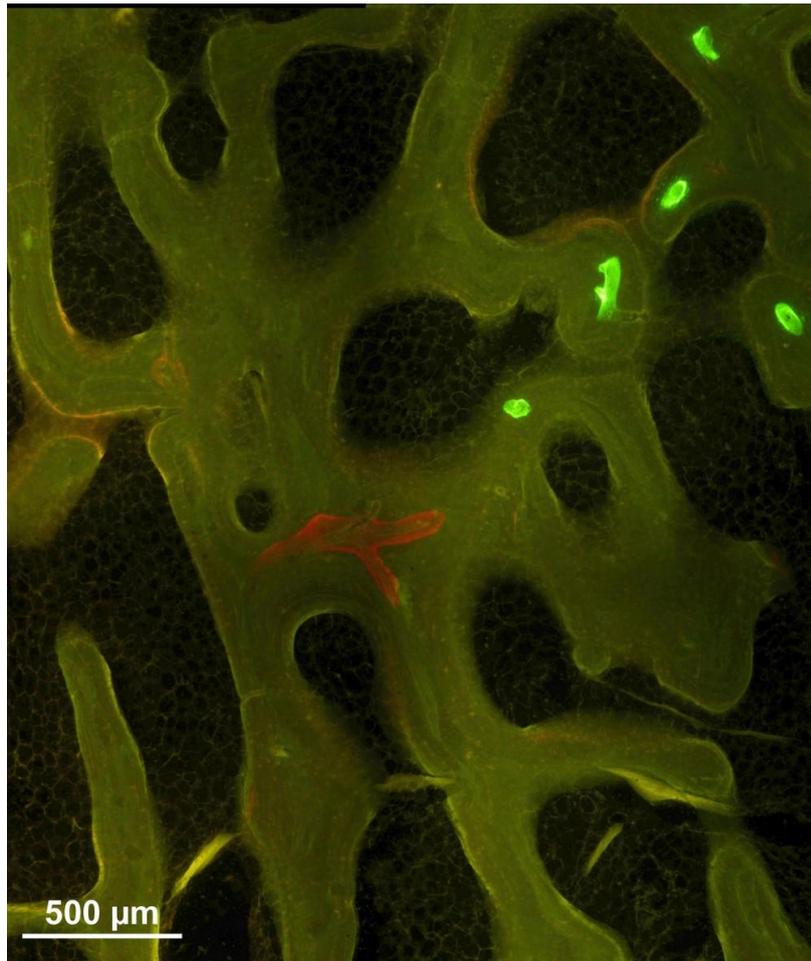


Figure 3. Intra-trabecular osteonal remodelling, labelled with Alizarin complexone (red), visualised using blue ($\lambda=470\text{nm}$) epifluorescence microscopy

On examining the relationship between the bone microstructure and bone turnover, as measured by fluorochrome-labelling, there were initially no positive findings when examining the data as a whole. Once divided into control and OVX groups for subgroup analysis, however, there were marked differences in the correlations seen within the two groups. Within the control sheep, increase in bone turnover along the trabecular surfaces was significantly correlated with increased bone volume fraction, connectivity density and trabecular number (Figure 4). Trabecular separation was also significantly reduced with increased labelling (Figure 4). Within the OVX group, these correlations were non-existent,

and in fact trabecular separation and connectivity density tend to inverse correlations; despite a greater level of bone turnover being present, as seen earlier in this section, the measurable features of bone microstructure appear to reach a plateau and then have no further alterations in response to bone turnover beyond this level. There were no significant correlations between the number of sites of intra-trabecular labelled lamellar bone present and alterations in the subchondral trabecular bone microstructure as measured by MicroCT, either when examining the data as a whole, or when divided into OVX and Control groups for more detailed analyses.

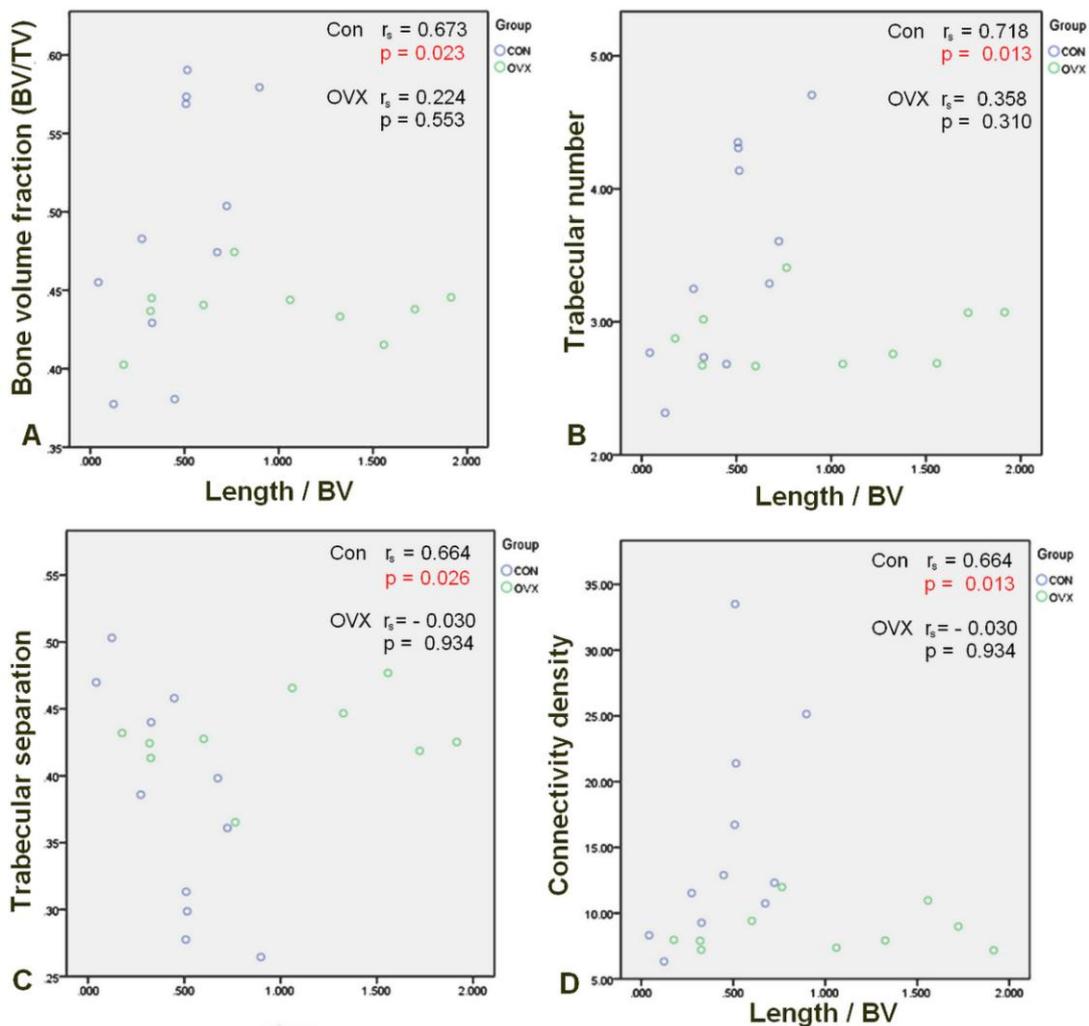


Figure 4. Length of sites of labelled bone per unit area of bone (Length / BV; mm/mm²) vs bone microstructure; (A) bone volume fraction (BV/TV), (B) Trabecular number, (C) Trabecular separation, (D) Connectivity density.

Next, bone turnover within the subchondral bone plate was assessed. This was significantly increased in the OVX group as compared to controls, with a higher total number of labelled sites visible in samples from OVX sheep, corrected for specimen width (2.35 vs. 0.85 # / mm; $p = 0.003$; Mann-Whitney U test; Figure 5). The OVX group had a slightly thinner subchondral bone layer than the control animals but not significantly so (6.59 vs. 8.32 mm, $p = 0.383$, t-test; 95 percent confidence interval for difference of means: -0.231 to 0.578). On examining the number of labelled sites per mm^2 of subchondral bone, the difference between the control and OVX groups was even more marked (4.68 vs. 0.69 # / mm^2 ; $p < 0.001$; Mann-Whitney U test; Figure 5).

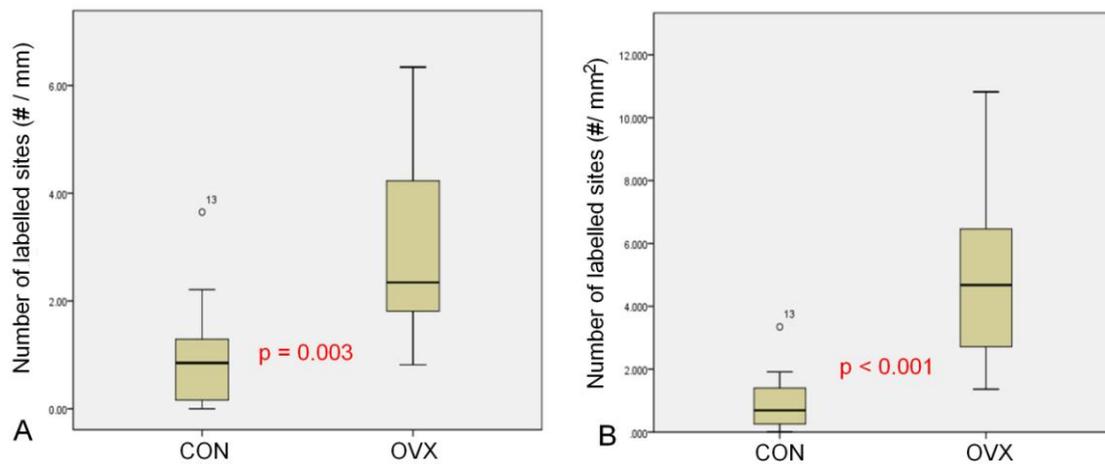


Figure 5. Number of sites of labelled bone, corrected for specimen width (A) and area (B).

As with the trabecular bone turnover, some unanticipated features were found when examining the labelled bone within the subchondral plate. A small number of secondary osteons within the subchondral plate contained two separate lamellae of labelled bone, despite these dyes having been administered 3 months apart (Figure 6).

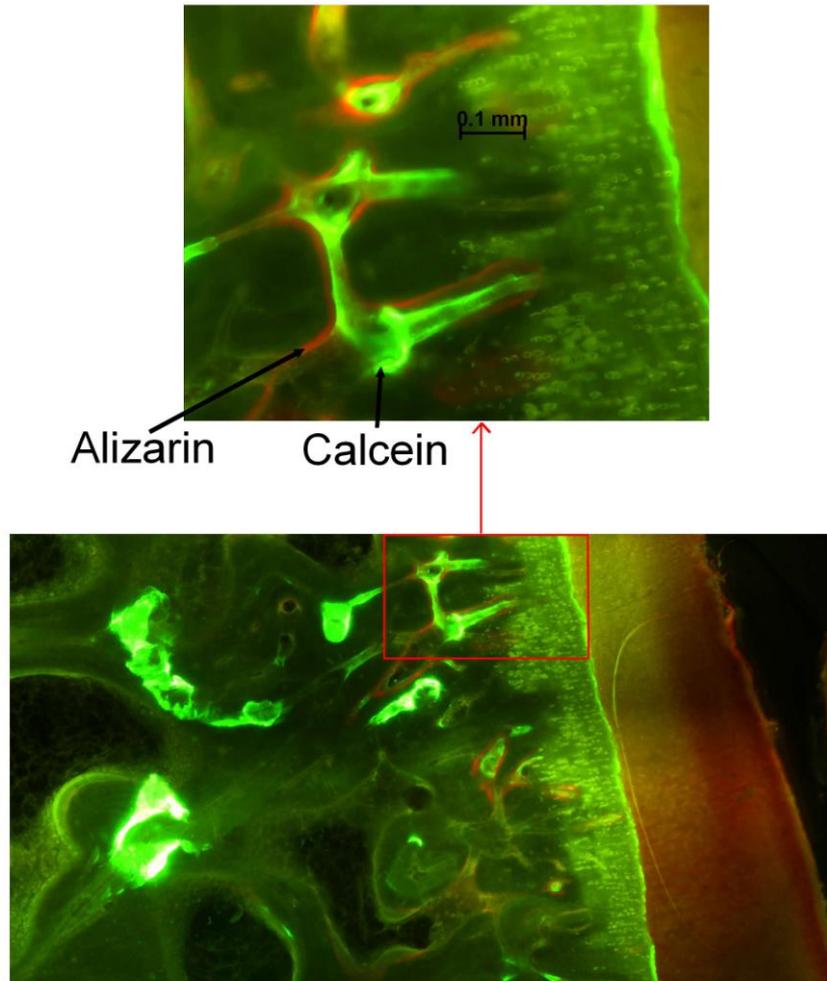


Figure 6. Double-labelled secondary osteons within the subchondral bone plate, visualised using blue ($\lambda=470\text{nm}$) epifluorescence microscopy

Discussion:

The majority of texts refer to cancellous bone remodelling as being hemiosteonal, with turnover only visible along the trabecular surfaces (Parfitt, 1994). However, in a study examining samples from 41 human iliac biopsies, Sato found an average of 0.55 (osteonal) channels per mm^2 of bone area (Sato et al., 1986). He noted a significant inverse correlation of channels present per unit of tissue area (TV) with increasing age in human iliac biopsies, but not per unit of bone area (BV). Another reference to trabecular osteons comes with

reference to removal of microfractures in cancellous bone. Boyde states these microfractures are not removed by immediate resorption, rather that surface bone deposition initially takes place, and that cutting cones subsequently develop within the (now thickened) trabecular plates (Boyde, 2003).

Our data show definitive evidence of intra-trabecular osteonal remodelling; while these were more prevalent within the OVX specimens, we found no correlation between the numbers of these osteons present and indices such as tissue or bone area. Sato did note a strong positive correlation between trabecular thickness and the number of channels, both in terms of tissue area and solid bone, but our data did not demonstrate this ($r_s = -0.066$, $p = 0.777$; $r_s = -0.237$, $p = 0.300$).

The more classically described hemi-osteonal bone turnover along the trabecular surfaces was also elevated following ovariectomy; the number of sites of labelled bone present per unit area of bone was significantly higher in OVX sheep as compared to controls. This is to be expected, given that oestrogen withdrawal by ovariectomy has previously been shown to result in substantial increases in basic multicellular unit (BMU) activation, which is responsible for bone remodelling, within 12 months (Kennedy et al., 2009b, Newman et al., 1995). However, labelled bone observed is not solely due to BMU activation; modelling is also continually occurring along the trabecular surfaces in the healthy skeleton. When correlations between bone turnover sites and bone morphometry were examined in the two subgroups, the control group showed strong correlations between increased length of labelled bone along trabecular surfaces and microstructural parameters such as bone volume fraction (BV/TV), connectivity density and trabecular number; this implies that the labelling is in newly deposited bone formed by modelling. However, the OVX groups showed no evidence

of any positive correlation between fluorochrome-labelling and the above parameters. In the early post-ovariectomy period, modelling is suppressed when osteopenia is developing (Flieger et al., 1998, Frost and Jee, 1992) and there is accelerated bone remodelling following oestrogen withdrawal (Kennedy et al., 2009b, Newman et al., 1995). While bone formation may increase, this rate is inadequate to replace the bone lost by resorption, leading to an elevation in the number and length of labelled sites present, but an overall loss of bone upon examination of the bone microstructure (Parfitt, 2004, Riggs and Melton, 2002) .

Bone turnover within the subchondral plate was also significantly increased within the OVX group as compared to controls, whether controlled for specimen width or area; this supports Parfitt's position that trabecular bone remodelling does not always exceed that found within the cortices (Parfitt, 2002). The majority of these osteons had a single fluorochrome present, but a small percentage of the subchondral osteons had two, typically alizarin complexone and calcein (administered at 12 and 24 weeks post-ovariectomy); in all cases the internal labelled lamellae surrounded the central canal. New bone may take up to 6 months to fully mineralise (Bilezikian et al., 2004, Burr, 2004); a delay in mineralisation of the innermost lamellae is the most likely explanation for the presence of two dyes within a single osteon, despite administration 3 months apart.

Increased bone turnover at other sites in the ovine skeleton has been shown to result in significant changes to the biomechanical behaviour of these bones (Kennedy et al., 2008a, Kennedy et al., 2008b). What then does this imply regarding the health of the adjacent joint? It has been suggested that in addition to the increased rate of observed osteopenia in specimens from patients diagnosed with SPONK (Mears et al., 2009), biochemical markers of bone turnover are also elevated (Berger et al., 2005). Microfractures within osteoporotic

subchondral bone are thought to be a possible aetiological mechanism for osteonecrosis of the knee (Lotke and Ecker, 1988, Yamamoto and Bullough, 2000). Subchondral insufficiency fractures secondary to osteopenia have been documented within the subchondral bone of the femoral head (Satku et al., 2003, Yamamoto and Bullough, 2000). Bone turnover has been investigated in the past with regard to its influence on fracture risk (Riggs and Melton, 2002). It has been demonstrated that accelerated bone resorption is associated with an increased incidence of osteoporotic fractures, independent of BMD (Hernandez, 2008, Meier et al., 2005). The presence of elevated subchondral bone turnover within the medial tibial plateau lends weight to the hypothesis that individual trabecular fractures may develop in this site, with deleterious consequences for the overlying joint. Interestingly, a recent study suggests that early treatment with Vitamin D and bisphosphonates, a standard treatment regimen for osteoporosis which reduces bone turnover, is beneficial in SPONK, with remission of both symptoms and pathological findings on MRI (Breer et al., 2012),

SPONK is often associated with osteoarthritis; rapid progression to osteoarthritis or joint destruction in both the hip and knee has been reported following diagnosis (Satku et al., 2003, Yamamoto and Bullough, 2000). Bone turnover in the underlying subchondral bone is increased in established osteoarthritis, as measured by scintigraphy, but few studies have performed direct evaluation of the remodelling rate by histological examination (Benske et al., 1988, Hayami et al., 2004, Sharif et al., 1995). Whether these changes precede or follow degradation in the overlying cartilage is unknown; the hypothesis that alterations in the mineralization or structure of the subchondral bone may be the initiating factor in the development of disease in the overlying cartilage is not new (Radin and Rose, 1986). Certainly once osteoarthritis is established, whatever the initiating cause, there is absolute

consensus that alterations in the subchondral bone do occur, which will then have a considerable effect on the stresses within the overlying cartilage (Burr, 2004).

In conclusion, this study shows significant alterations in bone turnover in both trabecular bone and within the subchondral bone plate at one-year post-ovariectomy. This is due to suppression of modelling and accelerated bone remodelling following oestrogen withdrawal. Remodelling of trabecular bone was due to both classically described hemi-osteonal and intra-trabecular osteonal remodelling. Osteons within the subchondral plate may have relatively late mineralisation of lamellae; the effect that this may have on the structural properties of the subchondral plate, or the health of the overlying cartilage, remains uncertain. However, the presence of both localised osteopenia and accelerated bone remodelling within the medial tibial plateau provide a possible mechanism for subchondral microfractures in the aetiology of SPONK. Further utilisation of the ovariectomised ewe may be useful for further study in this field.

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Conflict of interest statement:

None of the authors have any conflict of interests to report.

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