Temporal changes in bone composition, architecture, and strength following estrogen deficiency in osteoporosis.

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Temporal changes in bone composition, architecture and strength following estrogen deficiency in osteoporosis

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Abstract

Using an ovariectomised (OVX) ovine model, we provide an analysis of the timing of changes in bone following estrogen deficiency. The expression of genes known to regulate osteoclastogenesis, matrix production and mineralisation, as measured by real time RT-PCR, were all significantly increased by 12 months and increased expression was maintained through to 31 months post-OVX, compared to controls. FTIR spectroscopy confirmed that mineralised crystals were less mature than in controls 12 months post-OVX, and were even less so by 31 months. The mineral-to-matrix ratio was significantly reduced by 31 months while the ratio of mature to immature collagen cross-linking was initially increased at 12 months and subsequently reduced 31 months post-OVX. In contrast, trabecular number, thickness and separation were unchanged at 12 months. Significant reductions in the trabecular number and thickness and a significant increase in trabecular separation were observed 31 months after OVX. Most notably perhaps, these combined changes led to a significant reduction in the compressive strength of the trabecular bone after 31 months. The results indicate that there is an initial increase in bone turnover, which is accompanied by a change in bone composition. This is followed by a continued increase in bone resorption and relative reduction in bone formation, leading to deterioration in bone micro-architecture. Ultimately, these cumulative changes led to a significant reduction in the compressive strength of bones following 31 months of estrogen deficiency. These findings provide an important insight into the time sequence of changes during osteoporosis.
Keywords

Osteoporosis; Micro-architecture; Bone composition; Biomechanical properties; Animal model
Introduction

Postmenopausal osteoporosis is often referred to as high-turnover osteoporosis and occurs in women following menopause due to a decrease in estrogens [1]. Typically osteoporosis results in a thinning of the trabecular network, which leads to a reduction in bone volume fraction, increased trabecular separation and decreased trabecular number. These changes in architecture are instrumental in bringing about the significant reduction in bone strength, which is seen in osteoporosis. It has been shown that measures of bone architecture can explain a statistically significant proportion of the variability in bone strength [2].

A comprehensive study of bone from premenopausal, osteopenic and osteoporotic women found that trabecular bone volume fraction, trabecular number and trabecular thickness were reduced in osteopenic women compared to premenopausal women and these values continued to reduce in the osteoporotic group [3]. These changes have been explained by studying cytokines and growth factors that regulate bone cell activity during bone remodelling and osteoporosis. The receptor activator of nuclear factor Kappa B ligand (RANKL) and osteoprotegerin (OPG) produced by osteoblasts are dominant factors in osteoclastogenesis that lead to bone resorption [4].

The macro-scale biomechanical properties of bone have predominantly been shown to be reduced in osteoporotic bone. However, studies have shown that during estrogen deficiency, the yield strength and elastic modulus at the tissue level is increased relative to controls in an ovariectomised rat model of osteoporosis [5]. There is evidence that a change in the composition of the bone tissue occurs during estrogen deficiency. Studies have shown that estrogen deficiency reduces the mean bone mineral density [6, 7] while others have shown an increase in mineralisation [5]. Interestingly a recent study showed the distribution of tissue level mineral is more heterogeneous in osteoporotic femurs and also that these changes are
anatomically distinct and do not occur uniformly throughout the proximal femur [8]. In addition to changes in mineral, osteoporosis is accompanied by increased turnover of collagen and is associated with an overall reduction in the amount of type I collagen present in bone tissue [9, 10]. It has also been proposed that a polymorphism of the COL1α1 gene plays a role in the increased risk of fragility fracture during osteoporosis [11]. There are also reports that collagen cross-links may play an important role in determining fracture risk in aging and osteoporosis [12].

Osteoblasts are responsible for the production of non-collagenous proteins (NCP), such as osteocalcin (OCN) and osteopontin (OPN). Due to their high affinity for calcium phosphate during mineralisation, NCPs play a role in bone matrix organisation by acting as binding proteins and thereby act as mineral crystal nucleation sites on the organic matrix [13-16], but they also facilitate cell attachment during bone turnover [17]. However, there are little data in the literature on the effect of osteoporosis or estrogen deficiency on NCPs. That which does exist is conflicting as NCP content has been shown to be considerably reduced in osteoporotic bone when compared with age-matched or young controls [18], while other studies have reported that the levels of osteocalcin are increased following estrogen deficiency [19].

The objective of this study is to provide a comprehensive analysis of the timing of changes in bone microarchitecture, gene expression, composition of bone matrix, and mechanical properties of bone from estrogen-deficient animals. Specifically, trabecular microarchitecture was quantified by micro computed tomography 12 and 31 months post-ovariectomy. The expression of proteins known to regulate osteoclastogenesis (RANKL, osteoprotegerin, osteopontin), matrix production (COL1α1, COL1α2) and mineralisation (osteocalcin) were measured using real-time RT-PCR. FTIR spectroscopy analysis of matrix composition, including mineralisation and collagen cross-linking, was performed to determine how
changes in gene expression manifest themselves within the matrix. Finally, bone strength was measured to quantify the cumulative effect of these architectural and compositional changes on fracture susceptibility.

**Materials and Methods**

**Animal Study**

All animals were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*. Nineteen skeletally mature (> 4 years), mixed-breed ewes were randomly assigned into an ovariectomy (OVX, n=14) or control group (control, n=5) on which no OVX procedure was carried out. All procedures were carried out following institutional ethical approval and under an animal licence granted by the Irish Department of Health (B100/2443). Ovariectomy was performed through a ventral midline laparotomy under general anaesthesia with sodium thiopentone (Pentanol, Abbot Ltd, Sligo, Ireland) and maintained on 3-4% halothane (Merial Animal Health, Harlow, UK) in oxygen. Twelve months post-operatively, half of the ovariectomy group were sacrificed along with the 5 control animals. All remaining animals were sacrificed 31 months post-OVX.

**Micro Computed Tomography (μCT)**

A cylinder of trabecular bone was extracted from the left proximal femur of each animal using a diamond tipped coring tool (Cajero Ltd, Kent, UK). A specimen 7mm in diameter and 20mm in length was removed in the coronal plane from the fovea to the greater trochanter. To minimise deterioration of the bone over the duration of this study, all samples had minimal room temperature exposure and were stored in saline upon removal from the freezer prior to testing.
Trabecular microarchitecture at the proximal femur was analysed by µCT (µCT40, Scanco, Switzerland). The cylinders were wrapped in saline soaked gauze to ensure they remained hydrated during scanning. The scans were performed at a resolution of 8µm, at 70kVp and 114µA. A threshold value of 210 was selected based on the ability to adequately discriminate between bone and the other components present in the sample, i.e. air and marrow. Bone volume fraction (BV/TV), trabecular number (Tb.N. [mm$^{-1}$]), trabecular thickness (Tb.Th. [mm]) and trabecular separation (Tb.Sp. [mm]) were then quantified from the 3-dimensional reconstruction of the cylinder [20]. In calculating these parameters the cylinder was reduced by 1mm in diameter to allow for the build up of bone dust and fragments that were produced during the coring procedure.

**Mechanical Testing**

Following µCT scanning, the cylinders were press fitted into brass endcaps and these were attached to steel platens. Taking into consideration the depth of the endcaps, the dimensions of the cylinder (7mm in diameter and 20mm in length) were based on the work of Keaveny et al who recommended that cylinders of trabecular bone for uniaxial compression testing should have an effective gauge length to diameter ratio of 2:1 [21]. The samples were loaded in a servo-hydraulic materials testing machine (Instron, Bucks, UK) at a rate of 1mm/min using displacement control. Five preconditioning loads of 20N were applied before the cylinders were compressed to failure. The data were logged and plotted as a load-displacement curve. Calculation of the ultimate compressive strength was determined taking into consideration the effect of side artefacts, the concept that trabeculae around the perimeter are not attached at both ends and therefore do not contribute to the overall strength of the sample and should not be considered [22].
Real time Reverse Transcription Polymerase Chain Reaction (Real time RT-PCR)

Immediately following sacrifice, the right metacarpal of each sheep was snap frozen in liquid nitrogen and stored at -80°C prior to processing. On removal from the freezer the bone was initially cut into small fragments using a diamond saw (Struers, Germany) before grinding to a powder in a freezer mill (Spex Sample Prep 6750 Freezer Mill).

A sample of the powdered bone was placed in denaturation solution (Totally RNA Kit, Ambion) before the ribonucleic acid (RNA) was extracted using the phenol chloroform method, as per the manufacturer’s instructions, with the addition of glycogen (Ambion) to enhance RNA yield and aid recovery of the pellet. RNA concentration and purity (260/280 absorbance ratio) were determined using an atomic absorption spectrophotometer (Wallac Victor, PerkinElmer Life Sciences). Total RNA prepared using this method was found to be of sufficient quality to be used directly for real time reverse transcription polymerase chain reaction.

First-strand cDNA (complementary deoxyribonucleic acid) synthesis was performed with 1 µg total RNA (ribonucleic acid) from each sample, using a first-strand cDNA synthesis kit with Superscript III (Invitrogen; Carlsbad, CA, USA) and 250ng random hexamer primer (Geneworks, Adelaide, SA, Australia), in accordance with the manufacturer’s instructions. RANKL, OPG, COL1A1, COL1A2, OCN, OPN and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA expression were analysed by real time RT-PCR, using BioRad iQ SYBR Green Supermix (BioRad, Hercules, CA, USA) on a Rotor-Gene thermocycler (Corbett Research, Mortlake, NSW, Australia). The reactions were incubated at 94°C for 10 minutes for one cycle, and then 94°C (15 seconds), 60°C (30 seconds) and 72°C (30 seconds) for 45 cycles. All real time RT-PCRs were validated by the presence of a single peak in the melt curve analysis.
Two samples from each animal was analysed and real time RT-PCR reactions were carried out in triplicate for each sample. Relative levels of mRNA expression of the gene of interest between samples were quantified using the comparative cycle threshold (C_T) method. Briefly, the formula \( XN = 2^{-\Delta C_T} \) was used, where \( XN \) is the relative amount of target gene in question and \( \Delta C_T \) is the difference between the \( C_T \) of the gene in question and the \( C_T \) of the housekeeping gene, GAPDH, for a given sample.

**Fourier Transform Infrared (FTIR) Spectroscopy**

Powdered bone was added to potassium bromide and ground together in a mortar with a pestle. The powder was placed in a mould and compressed in a press at 10 tonnes to form a transparent specimen disc. The disc was placed into a Bruker Tensor 27 FTIR machine (Bruker Optik GmbH) and spectra obtained at a resolution of 4 cm\(^{-1}\) from 4000 to 400 cm\(^{-1}\). One powdered bone sample per animal was assessed and each spectrum was an average of 20 scans. On each spectrum the mineral-to-matrix ratio was assessed as the ratio of the area of the phosphate band to the area of the Amide I band. Crystallinity was calculated as the peak area of sub-bands at 1030 and 1020 cm\(^{-1}\). Collagen cross-linking (XLR) was calculated as the intensity ratio of sub-bands at 1660 cm\(^{-1}\), representing pyridinoline (PYD or mature) cross-links, and 1690 cm\(^{-1}\) representing dehydro-dihydroxylysinoornithine (deH-DHNLNL or immature) cross-links.

**Statistics**

Results are presented as mean ± standard deviation. To determine whether ovariectomy significantly altered the mRNA expression of bone turnover markers, a fully nested ANOVA was used (Minitab® Statistical Software). This analysis took into account the repeated measures made on each sample and the multiple samples analysed per animal. For the FTIR, \( \mu \)CT and mechanical testing data, results were tested for normality using the SPSS software.
package (SPSS Inc, Chicago, IL). ANOVA was used to determine statistical significance. A p-value of ≤ 0.05 was considered to be significant unless otherwise stated.

Results

Effects of Estrogen Deficiency on Trabecular Micro-architecture

Measurement of trabecular bone micro-architecture using µCT found that bone volume fraction (BV/TV) was significantly reduced following 12 and 31 months of estrogen deficiency (p ≤ 0.05) (Figure 1). However there were no significant changes in Tb.Sp, Tb.N or Tb.Th after 12 months. Significant changes in these parameters were not seen until thirty-one months post-OVX at which point there was an increase in Tb.Sp. and a reduction in Tb.N. and Tb.Th. (p ≤ 0.05).

Effects of Estrogen Deficiency on Gene Expression

The mRNA expression of each protein was normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and expressed relative to the 12 month controls. RANKL:OPG ratio was significantly increased relative to controls after 12 (p ≤ 0.05) and 31 months of estrogen deficiency following OVX (p ≤ 0.0001) (Figure 2). Longer term (31 months) estrogen deficiency resulted in an increase in RANKL:OPG mRNA expression relative to 12 months of estrogen deficiency (p ≤ 0.005).

Estrogen deficiency following OVX produced a significant increase in COL1α1 expression at both 12 and 31 months (p ≤ 0.001) relative to control animals (Figure 3). COL1α2 expression was also significantly increased in the estrogen-deficient animals at 12 (p ≤ 0.005) and 31 months post-OVX (p ≤ 0.05) relative to the control. Notably, expression of COL1α2 was
significantly reduced in the 31 month group compared to 12 months of estrogen deficiency (p ≤ 0.005).

Osteopontin expression was significantly increased following estrogen deficiency (p ≤ 0.001) relative to control animals at both time points (Figure 4). However, no significant difference was found between the estrogen deficient groups. Osteocalcin expression was also significantly increased with estrogen deficiency at 12 (p ≤ 0.05) and 31 months (p ≤ 0.001). It was found to be significantly higher in the 31 month deficiency group compared to the 12 month deficiency group (p ≤ 0.001).

Effects of Estrogen Deficiency on Matrix Composition

FTIR analysis found that crystallinity, or mineral maturity, was significantly reduced following 12 (p ≤ 0.01) and 31 (p ≤ 0.005) months of estrogen deficiency (Figure 5). Comparison between the two time points showed no difference in mineral maturity. Mineral to matrix ratio was not significantly reduced after 12 months of estrogen deficiency (p=0.1), however, a significant reduction was seen after 31 months (p ≤ 0.05). Collagen cross-linking was significantly increased 12 months post-ovariectomy (p ≤0.05) and then reduced to control levels after 31 months.

Effects of Estrogen Deficiency on Bone Strength

Static compression testing of the femoral trabecular bone cores showed no significant change in strength after 12 months of estrogen deficiency. However, a significant reduction in the compressive strength was seen after 31 months of estrogen deficiency (p ≤ 0.05) (Figure 6).
Discussion

This study used an ovine model of post menopausal osteoporosis to examine whether estrogen deficiency produces significant changes in bone matrix composition prior to, or subsequent to, significant changes in architecture and strength. We report for the first time that gene expression of markers for osteoclastogenesis, osteoblastogenesis and matrix constituents were all significantly changed at the earliest measured time point of 12 months post-OVX. Furthermore significant changes in the mineralised crystal maturity, mineral-to-matrix ratio and collagen cross-linking were detected at 12 months. These changes preceded significant architectural changes which did not occur until the later time point of 31 months post-OVX. Most notably perhaps, these combined changes led to a significant reduction in the compressive strength of the bones at 31 months following estrogen deficiency.

The involvement of estrogen in the prevention of osteoporosis and the maintenance of appropriate bone turnover in men and women has been well established [23, 24]. In early postmenopausal women and ovariectomised rats, estrogen withdrawal is associated with increased bone turnover in which resorption predominates over bone formation [25-27]. The production of the pro-osteoclastogenetic cytokine RANKL, by osteoblasts, is enhanced in estrogen deficiency [28] while OPG inhibits the maturation of osteoclasts [29]. In the current study, estrogen deficiency resulted in a significant increase in osteoclastogenesis as measured by RANKL:OPG expression ratio. Interestingly osteoclastogenesis was significantly greater after thirty-one months than after twelve months indicating increased bone resorption over time.

Collagen type I is the most abundant collagen found in the human body and is the main organic substance found in bone. At a molecular level, it is composed of a triple helix of two α1(1) chains and one α2(1) chain, which are encoded by COL1α1 and COL1α2 genes
respectively [30]. The current study found that estrogen deficiency was associated with elevated COL1α1 and COL1α2 expression, which is consistent with the increased collagen type I production seen previously [31]. However, the data presented here then show reduced collagen formation after thirty-one months relative to twelve months of estrogen deficiency. Unlike the RANKL:OPG data, which show bone resorption continuing to increase at the later time point, this result suggests that bone formation is slowing down over time. It is this combined effect of increased resorption and reduced formation that leads to the change in architecture in the estrogen deficient animals at thirty-one months.

It has been reported that synthesis of 10–15% homotrimers of α1, due to a polymorphism in the gene, may contribute to osteoporosis [32]. This results in collagen being formed, which no longer has the 2:1 ratio of α1(1):α2(1), rather there is an excess of α1(1) chains being synthesised and incorporated into the collagen molecule. In the current study the ratio of COL1α1:COL1α2 expression in the control group was 0.9 which is similar to levels measured in human studies previously [33]. Twelve months post-ovariectomy, this ratio had increased significantly to 610 and thirty–one months following ovariectomy this ratio had almost doubled to 1100. Previously it has been demonstrated using osteoblast-like cells that COL1α2 expression did not vary when cells were exposed to a mineralising media, whereas COL1α1 expression, which was initially high, progressively declined, leading to a decrease in the COL1α1:COL1α2 ratio concomitant with an increase in mineralisation [34]. The current study found that an increase in the COL1α1:COL1α2 ratio is associated with a reduction in mineralisation in the metacarpal bone. Our data also indicates that the variation in the COL1α1:COL1α2 was mostly attributable to elevated COL1α1 expression, as COL1α2 levels did not increase to the same extent following estrogen deficiency. This work suggests that
estrogen deficiency results in an over-expression of COL1α1 producing collagen, which is structurally, and potentially functionally, different from healthy type I collagen.

Another interesting result from this study was the increase in the ratio of mature collagen cross-links to immature after twelve months of estrogen deficiency followed by a reduction after thirty-one months. Using FTIR, collagen cross-linking (XLR) is calculated as the intensity ratio of pyridinoline (PYD or mature) cross-links to dehydro-dihydroxylys ionorleucine (deH-DHLNL or immature) cross-links. Newly formed collagen allows immature cross-links to form, resulting in a higher proportion of immature cross-links to mature cross-links [12]. As bone formation is high at twelve months a large number of immature cross-links are formed, over time these can convert to mature cross-links thus shifting the balance at the later time point. This shift in the ratio of mature to immature cross-links again poses an important question as to the quality of the collagen produced during estrogen deficiency. Further investigation of this and the partners of collagen that regulate collagen fibril structure, such as the small proteoglycans biglycan and decorin, are warranted.

There are conflicting data in the literature on mineralisation and mineral content during osteoporosis. Some studies have reported that mineral content is decreased [35, 36] while others have shown an increase [5, 37, 38]. The current study found no significant reduction in mineral-to-matrix ratio at twelve months although a strong downward trend was evident (p=0.1) however, by thirty-one months this reduction in ratio had reached statistical significance. This result supports previous work on this animal cohort which has shown through the use of quantitative backscatter electron imaging that mineral content is reduced following estrogen deficiency in the femur [6, 7]. However, another study has showed that changes do not occur ubiquitously throughout the proximal femur [8]. In the current study the twelve month estrogen deficient group also showed reduced crystallinity indicative of the increased turnover, which is confirmed by the gene expression data presented and previous
work on these animals [39]. In addition Figure 7 provides epifluorescence histological micrographs confirming the increased turnover in the ovariectomised group relative to the controls. After thirty-one months, crystallinity is unchanged, remaining lower than in the controls. This indicates a slowing down of bone turnover at the thirty-one month time point, which again we have previously seen in this same cohort of animals [40]. This is also consistent with the reduced bone formation as seen with the collagen gene expression data. Along with a reduced mineral content, these data indicate that the mineral, which is present following estrogen deficiency, is less crystalline than that found in healthy bone.

An interesting observation of this study is in the quantification of the changes in non-collagenous protein expression. Osteopontin is a protein that preferentially accumulates at mineralised tissue interfaces [41] and plays an important role in regulating osteoclastogenesis and is thereby often used as an indicator of bone turnover [42]. One recent study has suggested that over-expression of osteopontin is a risk factor for osteoporosis [43] and bone loss is suppressed in OPN-deficient mice following ovariectomy, despite an increase in osteoclastogenesis [44]. In the current study, we show for the first time that estrogen deficiency induced an increase in osteopontin expression which remained elevated over time and that these changes occurred prior to significant changes in bone microarchitecture.

In addition to being associated with increased mineralisation, osteocalcin is a terminal marker of osteoblast differentiation which delays crystal nucleation [45]. As such it has been suggested to play an inhibitory role during bone formation [46]. In the current study there was an approximate 10-fold increase in osteocalcin expression twelve months post-OVX while expression in the thirty-one month OVX group was almost 150 times that of the controls. As we have shown, bone formation is reduced by thirty-one months and this may indeed be as a result of the increased osteocalcin expression seen at this time point.
Another notable observation is that the expressions of the selected candidate genes was significantly altered after just twelve months of estrogen deficiency, and also coincided with changes in the mineral and matrix phases. In contrast to this, significant changes in trabecular number, thickness and separation were only detected after thirty-one months, and most interestingly these changes were accompanied by a significant reduction in the compressive strength of trabecular cores. While bone volume fraction was reduced twelve months post-OVX, this was likely due to the combined effects of non-significant reductions in trabecular number and thickness and increased separation. Mechanical strength was not altered at twelve months, thereby showing that although changes in bone composition precede bone architecture, these changes were insufficient to significantly reduce the mechanical properties of the bone.

One of the limitations of this study was the lack of a day 0 time point which would have allowed a direct comparison of changes over time. However, ethical constraints did not allow for the sacrifice of a group of animals at day 0. It must be noted that this study sought to understand the effects of estrogen deficiency over time rather than changes due to seasonal variation or age. However, we acknowledge that seasonal variation is a limitation of the ovine model. Ovariectomy procedures were carried out in November, when estrogen production is naturally at its highest. Therefore, in the absence of a Day 0 group, 12 month controls were used for comparison to both 12 month and 31 month OVX animals as these animals were sacrificed at the same time of year as the ovariectomy operations were performed. Another limitation of this study was the fact real time RT-PCR and FTIR were performed on compact bone samples from the metacarpal while μCT was performed on the proximal femur. While this is a drawback, the μCT data do show that estrogen deficiency is having a deleterious effect on the skeleton and thus proves the efficacy of the OVX sheep model. Therefore, the
data obtained from the total mRNA and on mineral and matrix composition can be assumed
to represent changes in bone following estrogen deficiency. In this, and other studies from the
group, we have shown increased bone turnover following estrogen deficiency in the ovine
model at a gene expression level and also using histology. However, we acknowledge that a
clinical measure of bone turnover would have been an interesting addition to this study.

In conclusion, this study provides new insights into the sequence of changes that occurs in
bone following estrogen deficiency. The results indicate that there is an initial increase in
both bone resorption and formation i.e. an increase in bone turnover, which is accompanied
by a change in bone composition. This is followed by a continued increase in bone resorption
and a relative reduction in bone formation, which leads to a deterioration in the bone micro-
architecture. Ultimately, these cumulative changes led to a significant reduction in the
compressive strength of the bones 31 months following estrogen deficiency. These findings
provide an important insight into the time sequence of changes during osteoporosis.

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Descriptors of Vertebral Cancellous Bone Architecture. J Osteoporos 641578


Fig. 1: Bone volume fraction (BV/TV) was significantly reduced following estrogen deficiency (a). Trabecular number and thickness (c and d) were significantly reduced after 31 months of estrogen deficiency and there was a significant reduction in trabecular thickness (b) at this time. (The level of significance denotes difference from the control group: #, p ≤ 0.05)
Fig. 2: RANKL:OPG mRNA expression was increased after 12 and 31 months of estrogen deficiency. Estrogen deficiency results in a continued increase in RANKL:OPG ratio over time (The level of significance denotes difference from the control group: #, p ≤ 0.05; *, p ≤ 0.0001: difference from 12 months OVX: **, p ≤ 0.005)
Fig. 3: COL1α1 (a) and COL1α2 (b) mRNA expression levels were both significantly increased following estrogen deficiency. The COL1α1: COL1α2 ratio was also significantly increased following estrogen deficiency. (The level of significance denotes difference from the control group for COL1 α 1; #, p ≤0.005: difference from control for COL1 α 2; #, p ≤ 0.005; *, p ≤ 0.05: difference from 12 month OVX; **, p ≤ 0.05; difference from control for COL1α1: COL1α2; #, p ≤0.005)

231x82mm (150 x 150 DPI)
Fig. 4: Both OPN (a) and OCN (b) mRNA expression (relative to GAPDH) were significantly increased following estrogen deficiency. (The level of significance denotes difference from the control group in OPN: A, p ≤ 0.001; in OCN difference from control: #, p ≤ 0.05; *, p ≤ 0.001; difference from 12 months OVX: **, p ≤ 0.001)
Fig. 5: FTIR spectroscopy found no significant difference in mineral to matrix ratio (a) after 12 months of estrogen deficiency, however, there was a significant reduction after 31 months. Mineral maturity was found to be significantly reduced following estrogen deficiency in both groups (b). Collagen crosslinking (c) was significantly increased after 12 months of estrogen deficiency before falling again (The level of significance denotes difference from the control group in Mineral-to-Matrix ratio: #, p ≤ 0.05: in Crystallinity, *, p ≤ 0.01, **, p ≤ 0.005: in XLR, #, p ≤ 0.05).
Fig. 6: The compressive strength of trabecular bone following 31 months of estrogen deficiency was significantly reduced (The level of significance denotes difference from the control group: #, p ≤ 0.05)

90x95mm (150 x 150 DPI)
Fig. 7: Transverse cross section of a metacarpal bone viewed under ultra-violet epifluorescence microscopy at X10 magnification. Intravenous fluorochrome markers (Calcein, calcein blue, oxytetracycline, xylene orange and alizarin complexone) were injected into the animals over the duration of the experiment, these markers labelled sites of new bone formation. High levels of bone turnover are visible after 12 months in (a) OVX bone while low levels are seen in (b) control bone. Histological data on these animals can be found in previous studies from our group [39, 40].