# Effects of Estrogen Deficiency on Vascularization and Mineralization in a Novel 3D Humanized Bone Model

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**INTRODUCTION:** During osteoporosis bone loss occurs, but 2D cell culture and animal studies have revealed that these changes are accompanied by fundamental changes in bone composition and cellular responses to mechanical loads [1,2,6-8]. Our recent research revealed the effect of estrogen deficiency on mineralization, [2] and osteoclastogenesis [3] in murine osteoblasts in 3D and 2D models. Vascularization is critical to bone formation and mineralization via the endochondral ossification process [3,4]. Estrogen regulates the lipid use of bone endothelial cells, whereas in low estrogen vascular aging and adipocyte accumulation occurs within bone, which might be associated with bone health [8]. However, the interaction between vascular and bone cells during estrogen deficiency is not fully understood. Thus, here we implement an advanced 3D vascularized and humanized bone model to provide a mechanistic understanding of changes in bone vascularization in estrogen deficiency, and understand how this process regulates bone composition.

**METHODS:** Mesenchymal stem cells (HBMSCs) were isolated from bone marrow (42-year-old female) under ethical approval and informed consent (Research Ethics Committee, University of Galway). HBMSCs (P5) were encapsulated in gelatin-mtgase (3% W/W) at $2 \times 10^6$ cells/ml and cultured in media for 21 days to develop a chondrogenic template (Fig 1A). At day 22, vascularization of the template was initiated by culturing the constructs within a hydrogel in which human umbilical vein endothelial cells (HUVECs) and HBMSCs (1:1) were encapsulated. For non-vascular constructs, only HBMSCs were added within the hydrogel. Both vascularized and non-vascularized groups were cultured for a further 21 days (until D42) in osteogenic differentiation media with 10nM 17β-Estradiol. At day 43, the constructs for both groups were cultured for a further 20 days (until D63) under either continued estrogen or estrogen withdrawal as follows: (i) Vascular-E, (ii) Vascular-EW, (iii) Non-Vascular-E, (iv) Non-Vascular EW. The constructs were analysed to determine vascularisation, osteogenesis and mineralization by histology (Alcian blue, Von Kossa), immunofluorescence staining for CD31, immunohistochemistry (Collagen I, X), biochemical assay (calcium content) and qRT PCR gene expression analysis for RANKL/OPG, DMP-1 and OPN.

**RESULTS:** ***Vascularization:*** Immunofluorescence staining for CD31 confirmed the formation of hollow vessel-like structures in the vascularized constructs by day 52 (Fig. 1B). There was a significant decrease in the fluorescence intensity of CD31 under estrogen withdrawal relative to those cultured under estrogen supplemented conditions (V-EW vs. V-E) by day 63 (Fig 1C, D). ***Osteogenesis:*** In non-vascularized groups, there was an increase in calcium content under estrogen withdrawal conditions (NV-EW vs. NV-E) at day 52, whereas in the vascularized groups this effect was observed by day 63 (V-EW vs. V-E), see Fig 1K. The calcium content was significantly higher in the vascular estrogen withdrawal group compared to the estrogen control (V-EW vs. V-E, Fig 1K), at day 63. The extent of mineral staining was highest in the vascular estrogen withdrawal group at day 63, which had distinct mineralized nodules (Fig 1E). By day 63, all constructs stained positive for Collagen I and X (Fig 1 F, L). The intensity of Col X was significantly higher in the vascular estrogen deficient group compared to the non-vascular group (V-EW vs NV-EW) by day 63 (Fig 1M). The intensity of collagen I was significantly lower in vascular estrogen deficient group compared to vascular estrogen supplemented group (V-EW vs V-E), at day 52. (Fig 1G). Gene expression for DMP-1 was significantly upregulated in the vascular estrogen deficient group compared to vascular estrogen supplemented at day 52 (Fig 1H). RANKL/OPG and OPN were significantly upregulated in the non-vascular estrogen deficient group compared to non-vascular estrogen supplemented and vascular estrogen deficient group at day 63 (Fig 1I, J).

**DISCUSSION:** The current study provides an *in vitro* 3D vascularized and humanized bone model that recapitulates estrogen deficiency representative of the osteoporotic bone phenotype. This work builds upon our previous studies that independently investigated (a) the effect of vascular cells on mineralization in 3D organoid models [3, 4], and (b) the effect of estrogen deficiency on mineralization and osteoclastogenesis in murine osteoblasts [2,5]. During endochondral ossification the vasculature infiltrates the lacunae and release chemokines to attract osteogenic cells and initiate bone formation. Our results confirm the formation of CD31+ distinct vessel-like structures and we report that estrogen deficiency reduces vascularization. Interestingly, a significant increase in mineralization and matrix formation was reported in the vascularized and estrogen withdrawal group. A recent *in vivo* study showed that estrogen deficiency decreases the number blood vessels and fatty acid metabolism, leading to increased number of adipocytes and decreased bone mineral [9]. Here we also report a reduction in the number of endothelial cells, but in contrast, we report that in our vascular model estrogen deficiency leads to an increase in the mineral deposition. This contradiction might be explained by the differential response of human and mouse bone cells in the presence of vascular cells during estrogen deficiency. Moreover, our model did not include mechanical stimulation, whereas *in vivo* the animals maintained activity. Collagen X is released by hypertrophic chondrocytes and plays a pivotal role in the endochondral ossification process [10]. Here we, saw an increase in collagen X in our vascular estrogen deficient constructs between day 52 and 63, which might explain the increased mineral deposition in these constructs under estrogen withdrawal. Our ongoing studies are further exploring the molecular interactions between vascular cells and osteoblasts during estrogen deficiency and mechanical stimulation.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Our findings show that estrogen deficiency decreases the number of endothelial cells but increases the mineral deposition, in an advanced 3D *in vitro* model. Clinically, this highlights a potential mechanism contributing to bone fragility in osteoporosis, where reduced estrogen levels could impact vascularization and mineralization dynamics, influencing fracture risk and treatment strategies.

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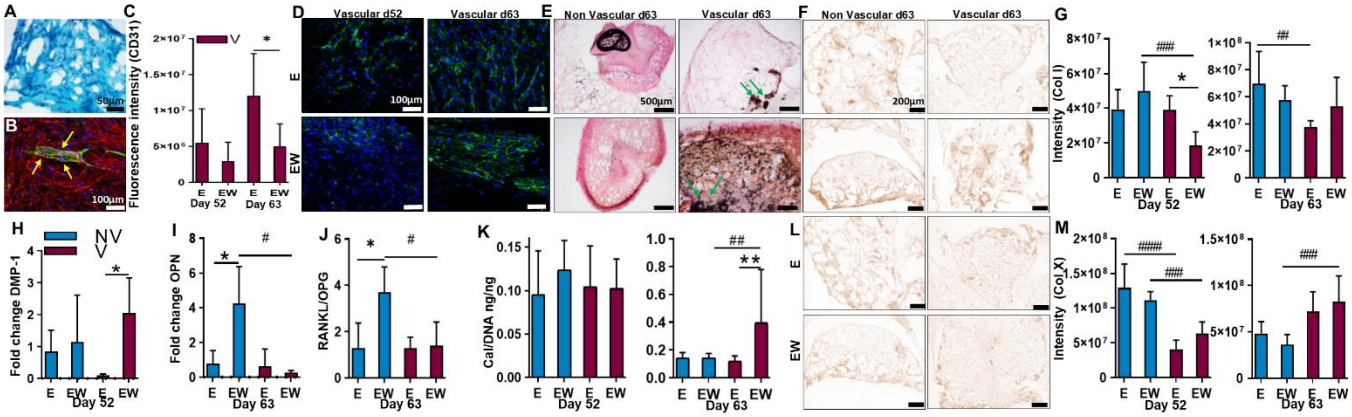


Figure 1: (A) Alcian blue staining of chondrogenic template. (B, C) CD31(green), Actin (red), DAPI (blue) immunofluorescence staining, yellow arrows indicate multicellular vessel. (D) Fluorescence intensity of CD31. (E) Von kossa staining at day 63, green arrows indicate mineral nodules. (F, G, L, M) Immunohistochemistry for Collagen I and X at day 63. (H, I, J) Gene expression for DMP-1, OPN and RANKL/OPG normalized to NV-E+. (K) Calcium content normalized to DNA. Significant differences ($p < 0.05$) indicated relative to E (*) and NV (#).