Regional assessment of male murine bone exposes spatial heterogeneity in osteocyte lacunar volume associated with intracortical canals and regulation by VEGF

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Abstract

The porous bone cortex comprises of a complex interconnecting network of intracortical vascular canals and osteocyte lacunae embedded within the bone mineral, with increases in cortical porosity reducing bone strength and increasing fracture risk. To date, our understanding of mechanisms coupling spatial vascular: lacunar network arrangements in the bone cortex is poorly understood yet it could be key in establishing cellular contributions to alterations cortical porosity evident with age. Using synchrotron radiation-based computed tomography we developed automated tools to characterise 3D spatial organisation and morphology of osteocyte lacunae and the bone vasculature at the tibiofibular junction (TFJ), defining posterior, medial, lateral, and anterior regions in male C57BL6 mice (n = 3). We also investigated a role for osteoblast derived VEGF in regulating the 3D spatial arrangement by conditional disruption of VEGF in osteocalcin-expressing cells (OcnVEGFKO versus WT, n = 3). Regional lacunar phenotypes were assessed by 3D distance mapping the lacunae organisation surrounding the vascular compartments within cortical bone including endosteal/periosteal surfaces or intracortical canals. Endosteal/periosteal surface associated lacunae were indistinct in size across posterior, medial, lateral and anterior regions. However, lacunae associated with intracortical canals were significantly larger but only within the posterior region. In the absence of VEGF, the increased lacunar volume associated with posterior intracortical canals was lost. Our results suggest that the influence of intracortical canals on lacunar volumes are spatially regulated and sensitive to locally produced growth factors such as osteoblast-derived VEGF.

Introduction

Skeletal pathologies including osteoporosis are characterised by increased cortical porosity leading to bones that are more prone to fracture, with 76% of the reduction in bone strength in aged bone attributable to altered cortical porosity (McCalden et al. 1993). The intracortical structure at microscopic scales comprises of intracortical canals and osteocyte lacunae (Schneider et al. 2009; 2013).

Intracortical canals house the bone vasculature, providing the bone cortex with blood supply to sustain bone cell viability and growth (Bonewald 2011). Throughout life the bone vasculature retains specialised structural and functional roles, with endothelial cell heterogeneity existing at both the organ (Marcu et al. 2018) and tissue level (Ramasamy et al. 2014; Kusumbe, Ramasamy, and Adams 2014; Ramasamy et al. 2016; Xie et al. 2014), suggesting that specific lineages of the bone vasculature may provide specific functions. In bone, specific vascular endothelial subtypes have been described and linked to angiogenic potential driving osteogenesis (Ramasamy et al. 2014; 2014; 2016; Wang et al. 2017; Zhu et al. 2019). In addition to functional variability, heterogeneity within intracortical canal distribution within specific bone regions has been documented in murine (Núñez et al. 2018; Schneider et al. 2007) and human (Perilli et al. 2015) bone. How region-specific arrangements of intracortical canals within bone facilitate bone cell survival remains unclear.

The second component of cortical microstructure is the lacuna-canalicular network (LCN), an interconnected network of voids (lacunae) and canals (canaliculi) (Bonewald 2011; Goggin et al. 2016). Within the LCN resides the osteocyte network, which comprises osteocytes within lacunae and their interconnected cell processes. Osteocytes are terminally differentiated osteoblasts that form the most abundant population of cells within bone (Bonewald 2011): despite their abundance, our understanding of their regulation in health and disease remains poorly understood. The location of osteocytes and the LCN within the mineralised bone cortex permits their primary functions as (1) mechanosensory cells (Tatsumi et al. 2007; Fritton and Weinbaum 2009) and (2) as regulators of bone (re)modelling (Bonewald 2011) in response to stimuli including mechanical loading (Zhao et al. 2022) and lactation (Qing et al. 2012). Mechanical stress compresses the bone cortex, driving interstitial fluid flow through the LCN (Bonewald 2011). Mechanical strains within the LCN are transduced by osteocytes into biochemical cues driving bone resorption by osteoclasts and bone deposition by osteoblasts (Bakker et al. 2001). Regional heterogeneity within the canalicular length, density and fluid

flow is observed across murine bone (van Tol et al. 2020), suggestive that functional heterogeneity exists within the LCN in response to heterogeneous strain distribution (Núñez et al. 2018).

In addition to initiating remodelling via osteoclasts and osteoblasts, osteocytes may remodel their local matrix through perilacunar remodelling (PLR). PLR allows deposition and resorption of the bone matrix surrounding the LCN (Qing et al. 2012; Lane et al. 2006). In response to lactation, the local matrix is resorbed through PLR to sustain systemic mineral content, leading to a transient expansion of the LCN and enlargement of osteocyte lacunae. However, an understanding of the heterogeneous morphology and distribution, and subsequent relation to the osteocyte and LCN function are not fully understood.

The proximity of osteocyte lacunae to intracortical canals within the bone matrix has prompted hypotheses that crosstalk exists between osteocytes and vasculature to couple angiogenesis and osteogenesis with suggestions that intracortical canal networks facilitate bone remodelling and development through direct interactions with osteoblasts and osteocytes (Goring et al. 2019) while preventing isolation of deeply embedded osteocytes (Núñez et al. 2018). Whereas proximity to intracortical canals may influence osteocyte distribution, the mechanisms underpinning heterogeneity within the distribution of intracortical canals and osteocyte lacunae are undefined.

We have previously resolved the regional distribution of intracortical canals at the murine tibiofibular junction (TFJ) in the context of ageing (Núñez et al. 2018). In aged murine bone, intracortical canal density was reduced exclusively in the posterior TFJ, highlighting the posterior TFJ as a region sensitive to the effects of ageing, providing a unique, pathologically susceptible anatomical site to interrogate the bone microstructure. Importantly, when the bone cortex was not regionally analysed, these age effects on the intracortical canals were not observed due to averaging across different cortical regions. While other studies have compartmentalised (partition in different regions, typically quadrants for long bones) the bone cortex for morphometric analysis (Núñez et al. 2018; van Tol et al. 2020; Uniyal et al. 2021; Schneider et al. 2007; Cole et al. 2022; Chiba et al. 2013), manual selection of regions may lead to inter- and intra-observer errors.

Although the assessment of the organisation of the LCN remain challenging due to its encasing within bone's mineralised matrix, techniques such as synchrotron radiation-based computed tomography (SR CT) permit non-destructive 3D high-resolution imaging of bone (Schneider

et al. 2007; Núñez et al. 2018; Goring et al. 2019). SR CT datasets may be segmented to isolate a negative imprint (Schneider et al. 2004) of the bone cortex, permitting the study of cortical porosity or the intracortical microstructure, such as the LCN.

Driven by the hypothesis that porosity within bone is heterogeneous and governed by region-specific spatial cues, we utilised SR CT to characterise intracortical canal and osteocyte lacunae distribution, morphology and spatial arrangements in the TFJ of murine bone. In this work we describe (i) an automated means to segment SR CT scans of the bone cortex (ii), a 3D distance mapping technique to evaluate how osteocytes and the bone vasculature are spatially organised, and (iii) how a pathological phenotype leads to disruption of this vascular: lacunar organisation.

Materials and methods

Animals

Male C57BL/6 wild-type (WT) mice were bred in house and maintained until 13 months of age. Post mortem, tibiae were dissected and fixed in 4% paraformaldehyde (pH 7.4 in PBS) for 48 h on rotation as described in (Núñez et al. 2018; Goring et al. 2019) and stored in 70% ethanol. Second-generation osteocalcin-specific VEGF knockout mice (OcnVEGFKO) were bred as described (Goring et al. 2019). Right tibiae were collected from 16-week-old male mice and prepared as above for 13m C57BL/6 tibiae. Use of animal tissue was carried out in compliance with the Animals Act 1986.

Synchrotron X-ray computed tomography

Synchrotron X-ray computed tomography (SR CT) was undertaken at Diamond Light Source (Harwell, UK) at Beamline I13-2 (Proposal 21843). In preparation for scanning right tibae were dehydrated and mounted in paraffin wax to prevent sample movement during scanning. The TFJ was selected as the field of view (1.8 x 1.2 mm^2) and for each scan 2000 projection images were acquired over a range of 360º. A photon energy of 18.5 keV was used with an exposure time of 500ms. Images were acquired using the pco.4000 camera and imaged with a x10 magnification to provide an isotropic voxel size of 1.65 µm. Datasets were corrected for ring artefacts and reconstructed using standard filtered back projection. The FOV was selected to incorporate a small region above the TFJ, with the first point of continuous contact between the tibia and fibula defined as the beginning of the TFJ. 500 slices were selected from this site of contact and used for analysis. The TFJ was set as the region of interest (ROI) to provide consistency in the analysed regions; further, the TFJ is an anatomical site consisting solely of cortical bone.

Alignment of the tibiofibular junction

From each scan, 500 slices of 8-bit greyscale image stacks starting at the TFJ were selected and imported into Fiji (Schindelin et al. 2012). Global iterative thresholding was used to convert greyscale images into binarized datasets, permitting separation of the bone cortex from soft tissue and air (Leung and Lam 1996). To standardise region separation across bone samples, the BoneJ moment of inertia function (Doube et al. 2010) was utilised to align the bone scan's principle axis to the image stack's x, y and z axes after which images were rotated -45° and canvas size adjusted to produce equal height to width ratio – e.g. 4000 x 4000 pixels.

Extraction of cortical porosity

The binary, aligned TFJ Image stacks were then duplicated and intracortical pores filled with a series of binary dilate functions. Upon complete closure of intracortical pores, the cortical mask was dilated to return the mask to the size of the original cortex. The cortical mask was inverted and combined with the thresholded stack through image addition, yielding cortical porosity as described (Núñez et al. 2018; Schneider et al. 2007).

Extraction of regional cortical porosity

Following TFJ alignment and isolation of cortical porosity, quadrants were identified via selection of coordinates within Fiji. For a canvas size of 4000*4000 pixels, quadrants of 2000*2000 were selected using "SPECIFY" function. "MAKE INVERSE" was used to select the remaining 3 quadrants and "FILL" used to fill these 3 quadrants with pixels with an intensity of 0 (black pixels, denoting empty space), leaving one quadrant with our pixels of interest (attributable to bone or cortical porosity). Specifying the upper left quadrant (0-2000 in x, 0-2000 in y) isolated the posterior, the upper right quadrant (2000-4000 in x, 0-2000 in y) isolated the lateral, the lower left quadrant (0-2000 in x, 2000-4000 in y) the medial, and the lower right quadrant (2000-4000 in x, 2000-4000 in y) isolated the anterior region of the TFJ.

Cortical total bone volume (Ct.TV) was calculated with BoneJ's particle analyser for each whole TFJ as well as each region. 3D visualisation of regional bone masks and regional cortical porosity was performed using Dragonfly 2020.2 (Object Research Systems). This segmentation technique allocated 28.20% (± 1.09%) of the total cortical volume to the anterior region, 35.27% (± 1.49%) to the posterior, 16.67% (± 0.23%) to the lateral and 19.85% (± 2.37%) to the medial region. Image processing workflows of the extraction of cortical porosity and alignment of bone cortices is illustrated in Fig 1A-B.

Pore quantification

BoneJ's particle analyser (Doube et al. 2010) was utilised to sort cortical porosity into noise (0 - 25 μm^3), osteocyte lacunae (25 - 2500 μm^3) and intracortical canals (> 2500 μm^3). Cortical porosity (Ct.Po (%)), canal number density (N.Ca/Ct.TV (mm-3)), lacuna number density (N.Lc/Ct.TV (mm-3)), canal volume density (Ca.V/Ct.TV (%)), lacunar volume density (Lc.V/Ct.TV (%)), average canal volume (⟨Ca.V⟩ (μm^3)), average lacunar volume (⟨Lc.V⟩ (μm^3)), were calculated in concordance with standard nomenclature for bone morphometry (Núñez et al. 2018; Goring et al. 2019; Bouxsein et al. 2010; Schneider et al. 2007; Hemmatian

et al. 2017). Lacunar volume distributions were visualised in Dragonfly. Osteocyte lacunae containing image stacks were imported and converted into multi-ROI datasets so that each osteocyte lacunae was treated as its own ROI. Object analysis tool was then used to quantify lacunar volume and mapped using the 'warm metal' LUT.

3D Lacunar distance mapping

Once osteocyte lacunae and intracortical canals were isolated an image subtraction (binary cortical mask subtract binary intracortical canal image stacks) was used to create a cortical mask perforated by intracortical canals (Fig 1E). The endosteal bone surface, periosteal bone surface and intracortical canals were designated as the sites of potential vascular supply.

This image was then inverted to convert the potential vascular surfaces as the regions of interest and imported into Dragonfly. A distance map was then created from the inverted intracortical canal mask to map the distance of each pixel within the bone cortex to the nearest surface of the inverted intracortical canal mask. Each pixel was then attributed a pixel intensity that directly relates to the distance from a vascular surface, in steps of the image resolution. A bone surface was attributed with a distance of '0', so received a pixel intensity of 0. Next, lacunae image stacks were imported into Dragonfly and converted to a multi-ROI so that every lacunae was treated as a separate entity. Each lacunae was superimposed onto the distance map, yielding a pixel intensities reflective of the pixels within that lacunae's distance from a bone surface. The smallest of these distances was then isolated to yield 'minimum lacunar distance' and coloured using the 'temperature' LUT. The minimum lacunar distance reflected the shortest distance between the lacunae and a potential vascular supply. This was completed for each lacunae and averaged to yield the mean minimum lacunar distance (Mm.Lc.D).

To determine the role of intracortical canals in the support of lacunae and influence on Mm.Lc.D the inverted cortical mask was also imported into Dragonfly and a distance map calculated between exclusively the endosteal and periosteal bone surfaces, in the absence of intracortical canals (Canal excluded distance mapping, termed 'Ca.Excl'). This was then compared to the lacunar distance mapping analysis including intracortical canals (Canal included distance mapping, termed 'Ca.Incl'). Should the minimum distance between and osteocyte lacunae and a potential vascular supply remain the same in both Ca.Incl and Ca.Excl analysis, then a bone surface is the nearest vascular supply and a lacunae is termed surface associated. Should canal inclusion alter the minimum lacunar distance then intracortical canals

reduce the distance between that osteocyte lacunae and a potential vascular supply and this lacunae is classed as canal associated.

In addition to Mm.Lc.D, Lacunae were binned based on their proximity to a bone surface; those within 25 µm, 50 µm, 75 µm, and >100 µm. The percentage of lacunae attributable to each of distance bins (%Lc.D.D) was then calculated and termed lacunar percentage distance distribution (%Lc.D.D). 100 µm was selected as the upper boundary for lacunar distance as the literature has reported that this is the maximum distance that an osteocyte can survive while relying solely on diffusion (Zahm et al. 2010).

Lacunar association with vascular supply

To determine which lacunae were associated nearby to intracortical canals or a bone surface for vascular supply the minimum distance was calculated in the Ca.Excl. and Ca.Incl. analysis for each lacunae and then compared. If the two values were identical for a given lacuna, then that lacunae was classified as surface-associated as removing the intracortical canals from the analysis had no effect on that lacuna's Min.Lc.D. If the distance differed between Ca.Incl. and Ca.Excl. distance maps, then intracortical canals were the nearest site of vascular supply and thus that lacunae was classified as intracortical canal-associated. This 'association filter' was applied to each lacuna in each region, and permitted the sorting of lacunae into two distinct populations; canal-associated lacunae, and surface-associated lacunae as illustrated in Fig. 1C. From this the percentage of lacunae associated with intracortical canals was calculated within each region and the distance reduced by intracortical canals. In addition to Min.Lc.D, ⟨Lc.V⟩ was calculated for each lacunae. Image processing workflows for 3D distance mapping of osteocyte lacunae positioning may be observed in Fig 1D and E.

Statistical analysis

To investigate the role of region on bone's microstructural characteristics, a one-way analysis of variance (ANOVA) was used with Tukey's multiple comparisons were conducted to assess statistical differences between groups as detailed in figure legends. For the assessment of cortical microstructural parameters in OcnVEGFKO, a paired two-way ANOVA employed to compare littermates. Values are shown as mean ± SD, and $p < 0.05$ was deemed statistically significant. All statistical tests were performed in GraphPad Prism 6.0 ("GraphPad Prism Version 6.0")

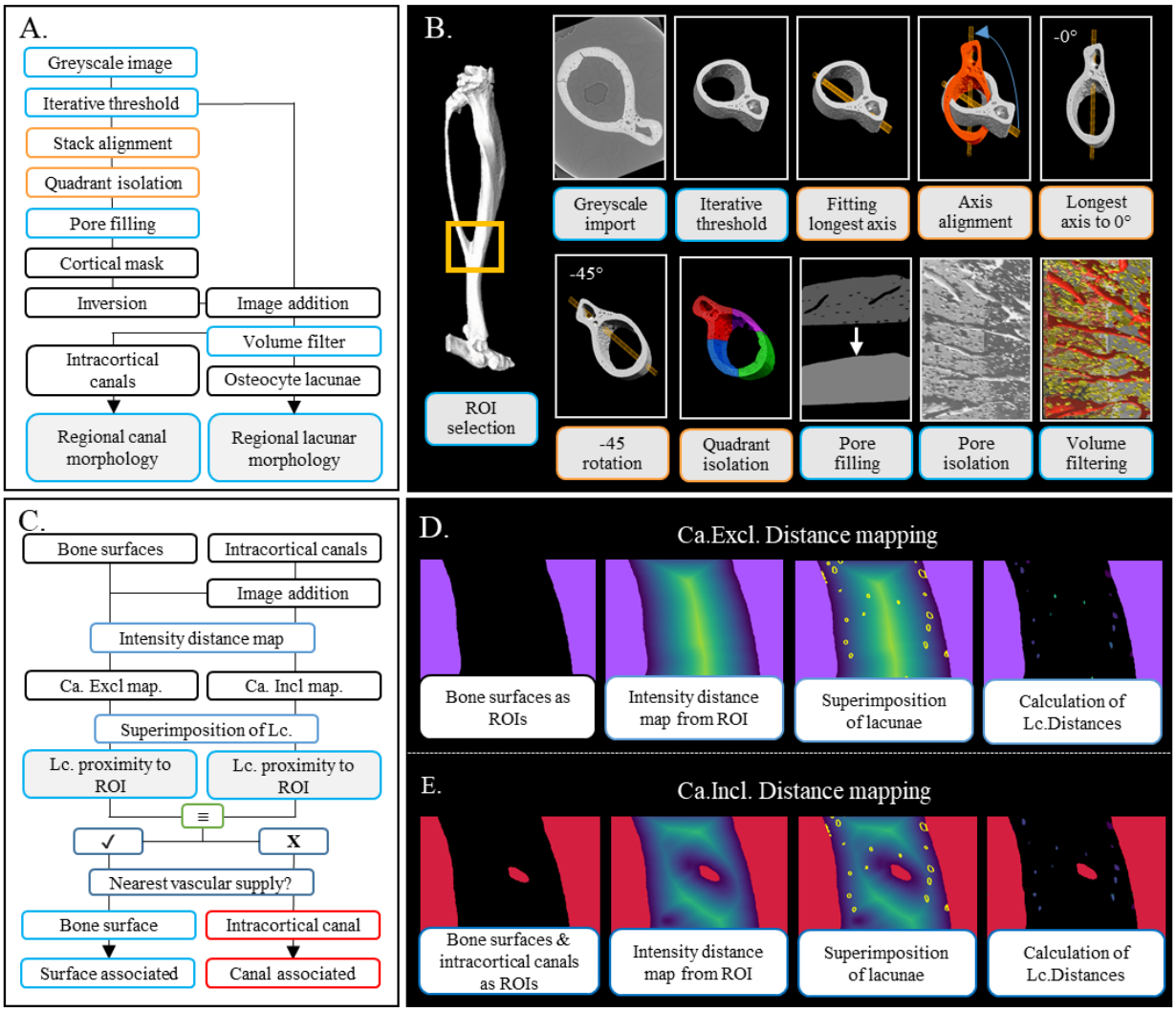

Fig. (1): Image processing workflow for automated regionalisation of the murine TFJ and the assessment of microstructural organisation within. (A) SR CT scans of the murine TFJ were imported into Fiji. Image stacks were aligned by fitting an ellipsoid to the TFJ, with the TFJ's longest axis manipulated to 0º. The aligned TFJ split into quadrants. Regional cortical porosity extracted and subject to a volume filter to discard noise (0-25µm^3) and to isolate osteocyte lacunae (25-2500 µm^3) and intracortical canals (>2500 µm^3). (B) Visualisation of image processing workflow described in (A). (C) Workflow for the assessment of osteocyte lacunae organisation in relation to bone surfaces or intracortical canals (D) and (E) visualise the workflow for Ca. Excl. distance mapping and Ca. Incl. distance mapping respectively.

Results

Regional distribution of cortical porosity in the murine cortex

Microstructural analysis of the tibiofibular junction (TFJ, Fig 2A) of 13-month-old male mice showed that global cortical porosity (Ct.Po%, Fig 2D) was 1.38% (± 0.01%) in line with previous observations (Willey et al. 2008) of the TFJ in WT mice. When assessed regionally, the posterior region possessed a significantly increased Ct.Po versus the anterior (+1.34%, p = 0.005), lateral (+1.08%, p = 0.017), and medial (0.93%, p = 0.036) regions (Fig. 2D, Table 1).

Intracortical canal density was higher in the posterior region versus the anterior (+65.6%, p = 0.042), lateral (+71.7%, p = 0.030), and medial (+68.7%, p = 0.037) regions (Fig, 2E, Table 1), while no significant regional differences were observed in canal volume density and average canal volume (Table 1).

Osteocyte lacunar number density (N.Lc/Ct.TV, mm^3, Fig. 2F, Table 1), average volume (⟨Lc.V⟩ , Fig. 2G, Table 1) and volume density (Lc.V/Ct.TV, Table 1) were also calculated within each region of the TFJ. Lacunar number density was higher in the lateral region than in the anterior region (+19%, p = 0.046). Lacunae were larger in the posterior region than in the anterior (+48.1%, p = 0.004) and lateral (+37.2%, p = 0.019) regions, while medial lacunae were also larger than anterior lacunae (+42.1%, p = 0.180). Lc.V/Ct.TV was also higher in the posterior and medial regions than in the anterior region (+52.8% p = 0.003 and +50%, p = 0.007, respectively). The correlation of increased intracortical number density and large osteocyte lacunae within the posterior region motivated use of 3D distance mapping techniques to assess the organisation of osteocyte lacunae surrounding intracortical canals between different regions.

	Region				Anova
Parameter	Posterior	Anterior	Lateral	Medial	*p*- value
Ct.Po (%)	2.22 ± 0.62	0.88 ± 0.13	1.14 ± 0.09	1.29 ± 0.19	**0.005**
Ca.V/Ct.TV (%)	0.97 ± 0.53	0.29 ± 0.064	0.29 ± 0.096	0.11 ± 0.03	**0.050**
N.Ca/Ct.TV (mm)	233 ± 92	76 ± 18	65 ± 2	71 ± 5	**0.020**
⟨Ca.V⟩ (μm^3)	37765 ± 12526	40322 ± 10320	43674 ± 14626	16417 ± 5565	0.144
Lc.V/Ct.TV (%)	1.25 ± 0.13	0.59 ± 0.16	0.85 ± 0.020	1.18 ± 0.22	0.003
N.Lc/Ct.TV (mm-3)	19172 ± 685	17345 ± 1950	20901 ± 337	20235 ± 596	**0.050**
⟨Lc.V⟩ (μm^3)	653 ± 47	339 ± 64	410 ± 15	585 ± 130	**0.003**

Table 1. Regional microstructural morphometric measures for cortical regions within the murine TFJ. Values indicate mean value ± SD with p-values < 0.05 denotes in bold, n = 3.

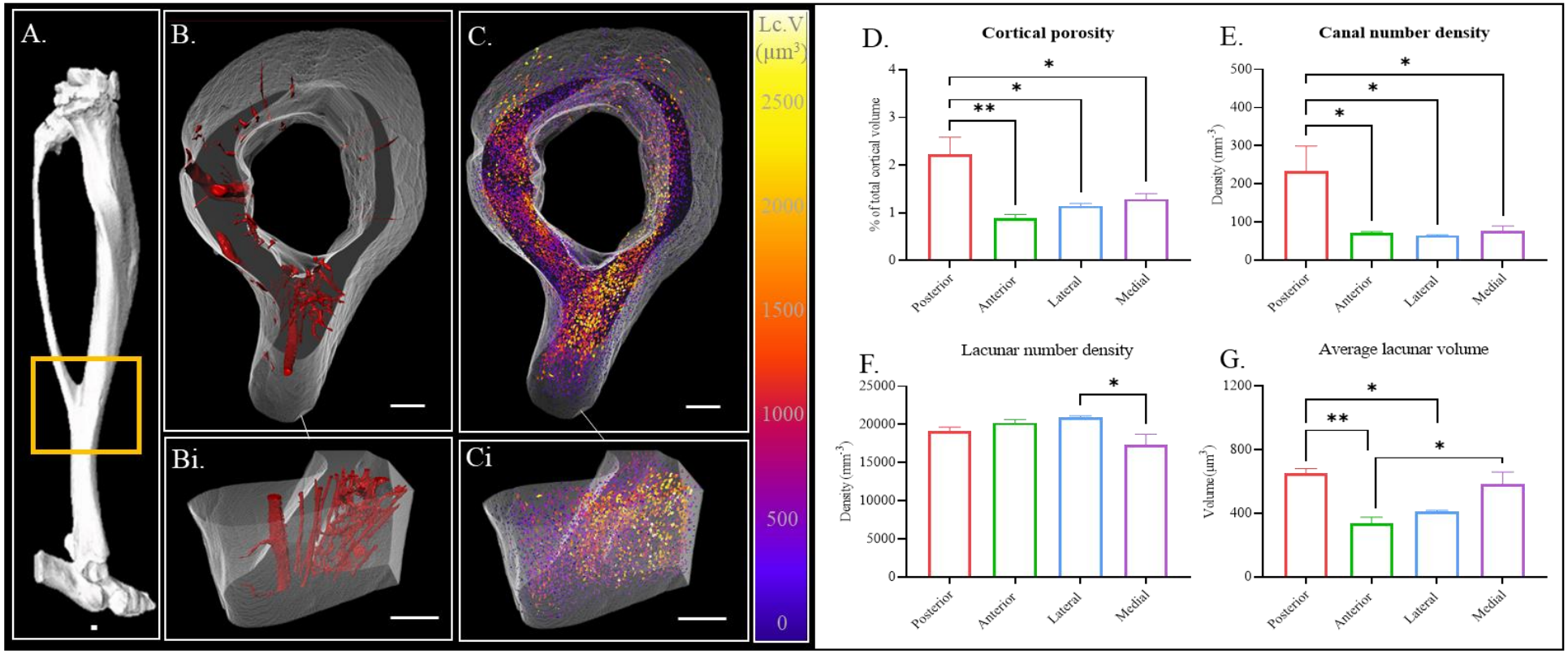

Fig. (2): Regionalisation of murine cortical bone reveals region-specific microstructural properties. Regional microstructure was quantified at the TFJ (A) and separated into intracortical canals (B) and osteocyte lacunae (C). The posterior TFJ is more porous than other regions (D). The number density of intracortical canals (E) varies between regions, with the posterior TFJ possessing more intracortical canals than other regions: visualised in (Bi) and quantified in (E). Isolation of osteocyte lacunae finds that although the differences in lacunar number density between regions are minimal (F), average lacunar volume varies between regions with the posterior TFJ possessing large lacunae as shown in (Ci) and quantified in (G). Large lacunae are colour in yellow and orange. *Data shown as mean ± SD, * = p<0.05, ** p<0.01. Scale bars represent 200µm.*

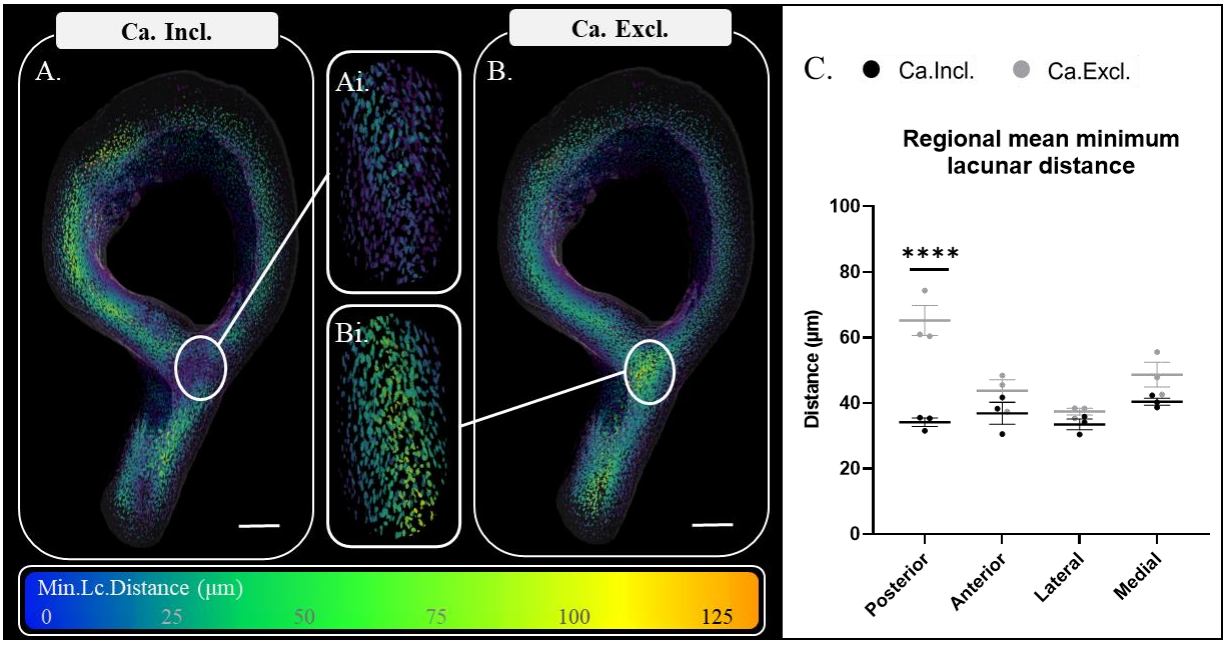

Fig. (3): Posterior intracortical canals reduce the proportion of lacunae isolated deep within the cortex. Spatial mapping of mean minimum lacunar distance (Mm.Lc.D) from both bone surfaces and intracortical canals (B, Ca.Incl) and with canals excluded from the analysis (B, Ca.Excl). Removal of intracortical canals increased the Mm.Lc.D in the posterior region but not in any other region (C). Scale bars denote 200µm. *Values reported are mean ± SD.* * denotes $p<0.05$, ** $p < 0.01$, *** $p <0.001$.

3D distance mapping of osteocyte lacunae around intracortical canals

3D distance mapping was used to assess the organisation of osteocyte lacunae surrounding potential vascular supplies within bone; that is, endosteal/periosteal surfaces and intracortical canals within each cortical region. Global analysis revealed that the mean minimum lacunar distance (Mm.Lc.D) is (36.2 µm ± 0.99 µm), while regional analysis found no significant difference between regions (anterior 36.9 µm ± 4.7 µm, posterior 34.2 µm ± 1.8 µm, lateral 33.5 µm ± 2.3 µm, medial 40.4 µm ± 1.5 µm) as assessed by a one-way ANOVA ($p = 0.139$, Fig. 3C, grey). 49.3% (± 1.57%) of lacunae were localised within 25 µm of a bone surface or intracortical canal, while a minority of lacunae (6.4% ± 0.5%) were at a distance of >100 µm from a possible vascular surface. To investigate potential regional differences in the association of osteocytes with different vascular sources, distance maps quantifying lacunar organisation were computed in two ways: 1) in relation to both bone surfaces and intracortical canals (Ca.Incl distance maps, Fig 3A) and 2) solely in relation to bone surfaces (Ca.Excl distance maps, Fig 3B) and 2). This enabled comparison of the minimum lacunar distance with (Ca.Incl) and without (Ca.Excl) intracortical canals, allowing identification of populations of osteocyte

lacunae closely associated with intracortical canals and those which would be isolated should intracortical canals not be present. Global analysis of Ca.Excl distance maps compared with Ca.Incl maps show that intracortical canals reduce global Mm.Lc.D from 48.8 µm (± 1.9 µm) to 36.23 µm (± 0.9, $p = 0.002$). This finding was specific to the posterior region with intracortical canal inclusion in distance maps reducing Mm.Lc.D from 60.9 µm (± 6.4 µm) in Ca.Excl distance maps to 34.2 µm (± 1.8 µm) in Ca.Incl distance maps ($p < 0.0001$). For the anterior ($p = 0.346$), lateral ($p = 0.81$) and medial ($p = 0.19$) regions canal inclusion no significant effect was detected (Fig. 3C). Further quantification of the number of isolated lacunae was completed through calculation of the proportion of lacunae found >100 µm from a bone surface. In the posterior region in Ca.Excl maps 26.8% (± 2.9%) were >100 µm from a potential vascular supply, while Ca.Incl greatly reduced this (3.4% ± 0.6%, $p < 0.0001$, Supp Data 1).

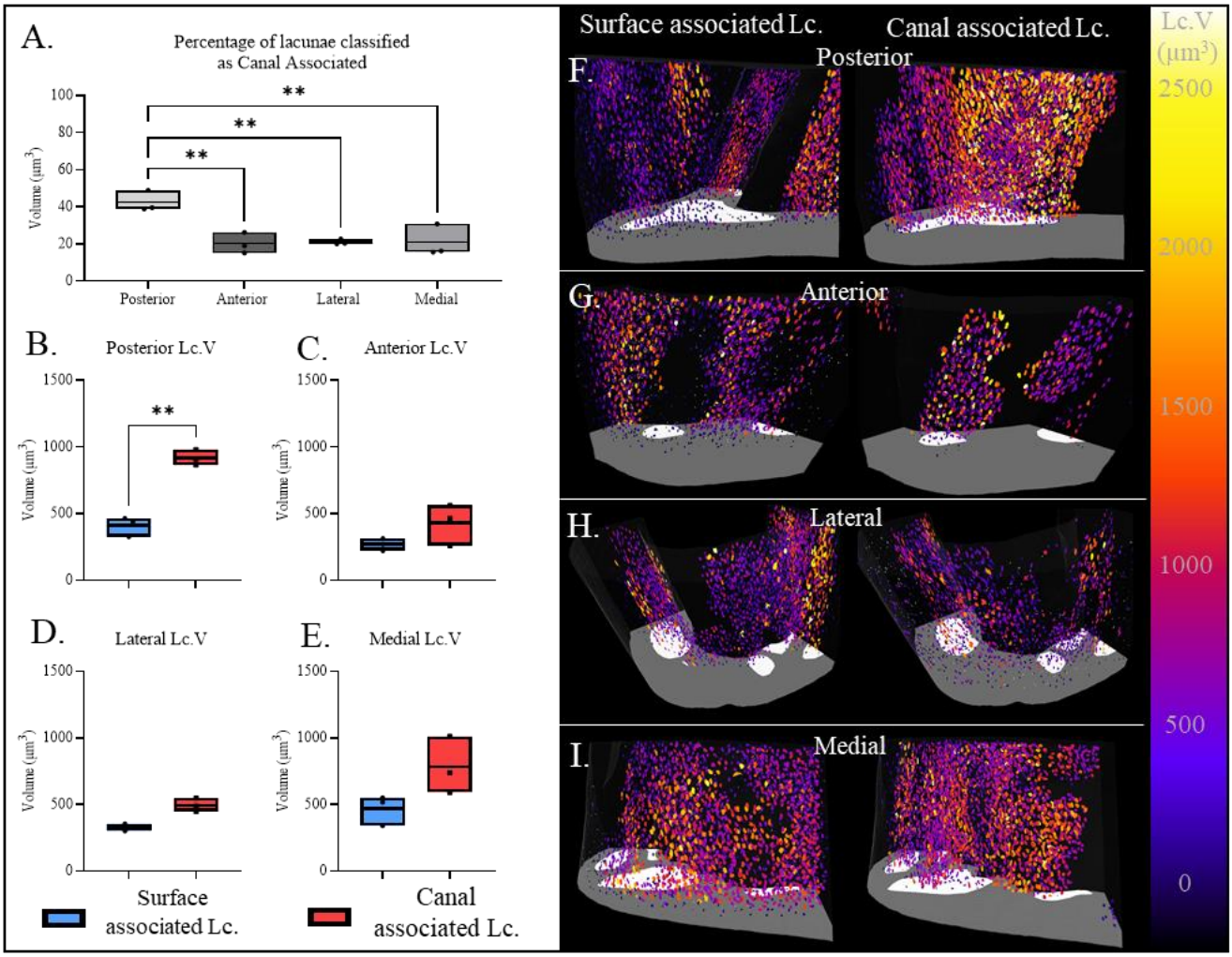

Fig. (4): Lacunae associated with intracortical canals are larger than surface lacunae in the posterior region (above). (A) Quantifies the proportion of lacunae defined as canal associated within each region, pore associate with canals versus surface in the posterior region versus other regions. The mean volume of canal and surface associated lacunae was calculated in the posterior (B), anterior (C), lateral (D) and medial (E) regions. 3D visualisation of canal and surface associated osteocyte lacunae volume distributions in the posterior (F), anterior (G), lateral (H) and medial regions (L). Larger lacunae are coloured in orange/yellow and smaller in blue/purple. Data is shown as mean ± SD and assessed by a one-way ANOVA with subsequent post hoc analysis. * denotes $p<0.05$, ** $p < 0.01$, *** $p <0.001$.

Canal-associated lacunae are largest in posterior region

To determine whether the abundant intracortical canals in the posterior TFJ support the large osteocyte lacunae in this region, lacunar populations were separated into canal-associated and surface-associated lacunae as illustrated in Fig. 1C. The posterior TFJ had more canal-associated lacunae (42.4% ± 4.5% of total lacunar number) than the anterior (20.1% ± 4.6%, $p = 0.007$), lateral (21.0% ± 1.2%, $p = 0.009$), and medial (20.9% ± 7.0%, $p = 0.009$) regions (Fig. 4B).

In the posterior region canal-associated lacunae (919 μm^3 ± 48 μm^3) were larger than surface-associated lacunae (411 μm^3 ± 60 μm^3, $p < 0.001$, Fig. 3D). In the anterior (Fig. 4E), lateral (Fig. 4F) and medial (Fig. 4G) regions, no significant difference was found in the average lacunar volume between canal-associated and surface-associated lacunae. Further, posterior canal-associated lacunae (919 μm^3 ± 48 μm^3) were larger than those in the anterior (429 μm^3 ± 126 μm^3, $p = 0.010$) and lateral regions (494 μm^3 ± 42 μm^3, $p = 0.022$).

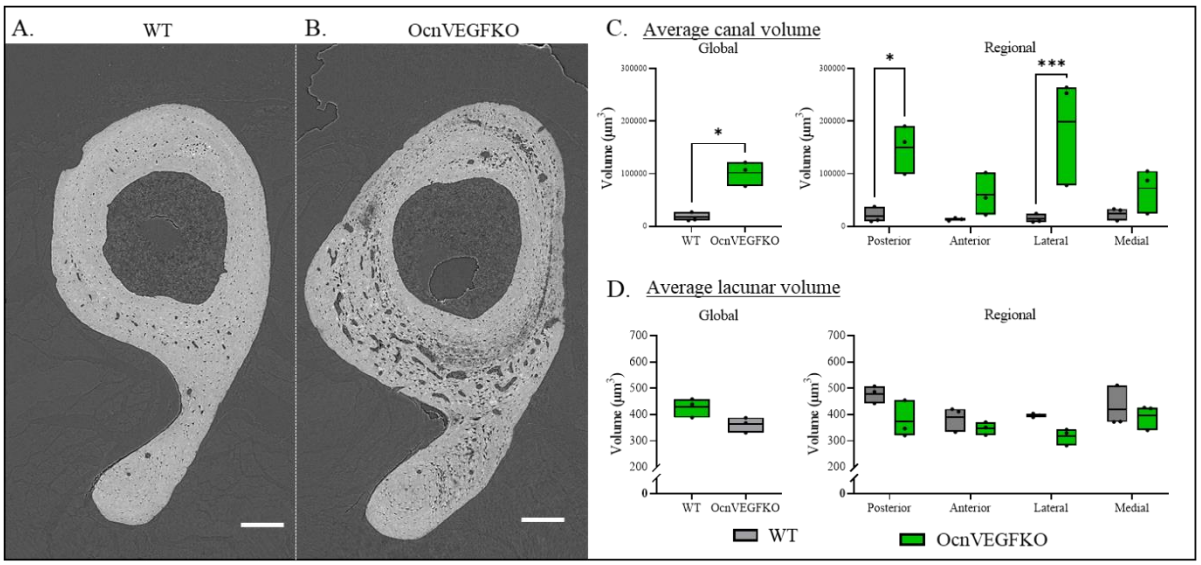

Fig. (5): Conditional VEGFKO deletion disrupts intracortical canal volume in a region specific manner. High resolution SR CT visualises male WT (A) and OcnVEGFKO (B) cortical bone at the TFJ, scale bar = 200µm. OcnVEGFKO mice possess larger intracortical canals (C) which upon regional assessment is localised to the posterior and lateral TFJ. Average lacunar volume is unaffected in OcnVEGFKO bone (D). N=3 WT and OcnVEGFKO, with littermates matched through paired comparisons. Data is presented as mean ± SD *$p < 0.05$, ***$p < 0.001$ using two-way ANOVA.

Effect of VEGF deletion on vascular: lacunar spatial arrangements

To study the nature of lacunar-vascular associations the OcnVEGFKO model was utilised. Disruption of VEGFKO in male mice leads to a highly vascularised TFJ (Goring et al. 2019). Male OcnVEGFKO cortices were highly porous (Fig. 5A-B) and heavily vascularised (Fig. 5C) with no significant changes to mean osteocyte lacunar volume (Fig. 5D).

Osteocyte lacunae were separated into canal-associated and surface-associated in wildtype (WT) (Fig 6A-Aiv) and OcnVEGFKO bone (Fig. 6B-Biv). There were no differences in the volume of surface-associated lacunae (Fig. 6C), and no difference in global canal-associated lacunae volume. Regionalisation of bone cortices revealed that in WT bone posterior canal-associated lacunae (564 μm^3 ± 42 μm^3) were larger than surface-associated lacunae (439 μm^3 ± 11 μm^3, $p = 0.034$). Meanwhile in OcnVEGFKO bone we did not find any significant difference in average volume between canal-associated lacunae (346 μm^3 ± 49 μm^3) and surface-associated lacunae (342 μm^3 ± 22 μm^3, $p = 0.120$, Fig. 6E), suggesting that loss of VEGF signalling by osteocalcin expressing cells prevents the formation of distinct, large lacunar populations.

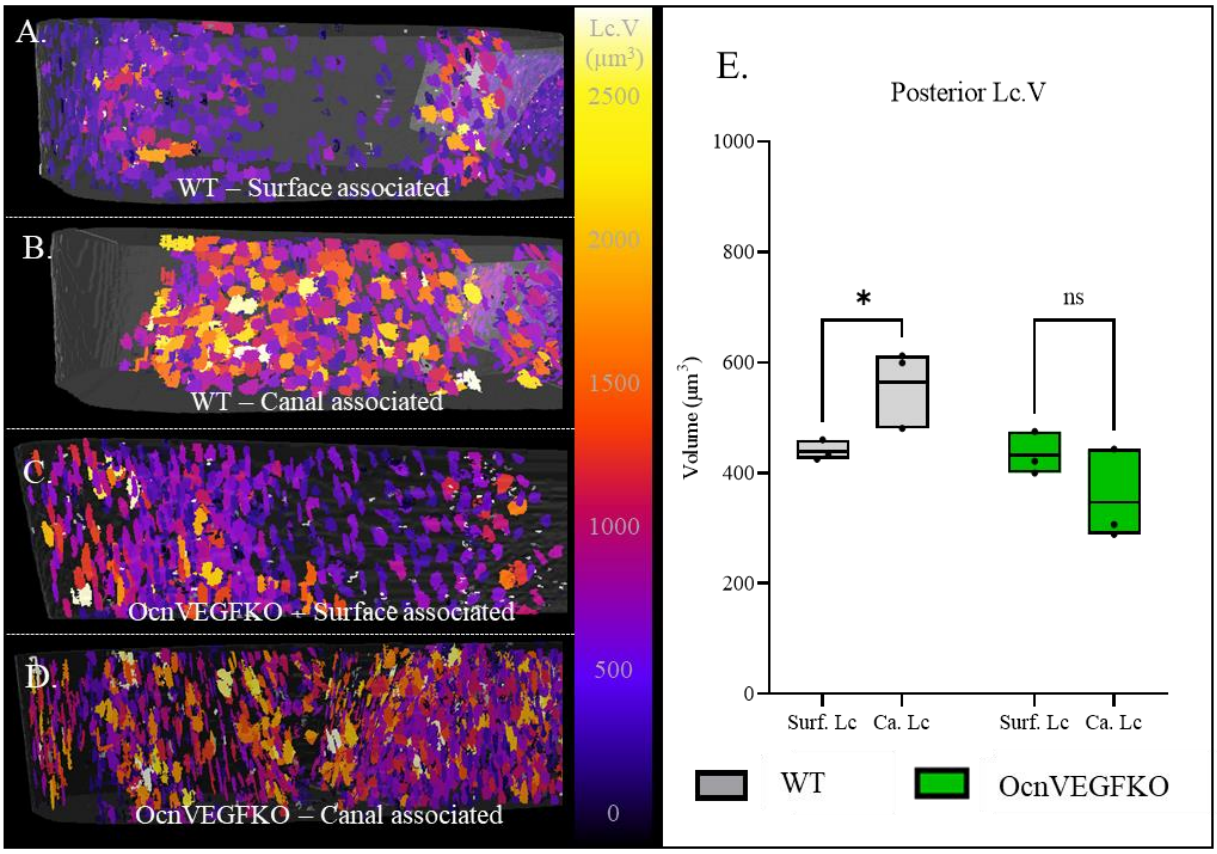

Fig. (6): Conditional VEGFKO deletion leads to a homogenous population of osteocyte lacunae in the posterior region. 3D visualisation of surface associated (A) and canal associated (B) osteocyte lacunae within the posterior TFJ of 16 week old male mice WT mice (n=3) and OcnVEGFKO mice (C.D respectively) with large lacunae shown in yellow and white and small lacunae in purple. (E) Quantification of average Lc.V between WT and OcnVEGFKO mice. Data is shown as mean ± SD and assessed by a two-way ANOVA with subsequent post-hoc analysis. * denotes $p<0.05$, ** $p < 0.01$, *** $p <0.001$.

Discussion

This study provides evidence that the cortical microstructure is heterogeneous and that a population of large osteocyte lacunae surround intracortical canals in a region-specific, VEGF-dependent manner. Specifically, SR CT was utilised to depict the bone microstructure in 3D at the murine TFJ. Upon development of an automated regionalisation tool, we identified the posterior region of the TFJ as a site possessing abundant intracortical canals and large osteocyte lacunae. By employing 3D distance mapping techniques we assessed the spatial organisation of osteocyte lacunae surrounding intracortical canals, and we could show that large osteocyte lacunae can be specifically found around the abundant intracortical canals within this posterior region. Notably, this distinct population of canal-associated, large lacunae within the posterior TFJ did not form in a murine model of conditional VEGF-knockout. Thus, this study (i) identifies a subset of osteocyte lacunae that appear closely coupled to the intracortical vasculature and (ii) reveals that the formation of this canal-associated lacunae sub-population is regulated by VEGF.

Regional assessment of cortical bone is commonly used to probe region specific alterations to the bone cortex utilising both 2D (Cole et al. 2022; van Tol et al. 2020) and 3D imaging modalities (Núñez et al. 2018; Uniyal et al. 2021; Schneider et al. 2007; Chiba et al. 2013). However, the use of manual segmentation for regional selection lacks reproducibility and thus consistency between studies. This work describes an automated technique in open-source software that enables accurate and reproducible regionalisation of the murine bone cortex, applicable to large 3D datasets. Our proposed method has been applied to separate the C57BL/6 TFJ into posterior, anterior, lateral, and medial regions. Prior regional assessment of bone has confirmed that specific cortical regions are prone to deterioration (Núñez et al. 2018; van Tol et al. 2020), with the posterior TFJ susceptible to the effects of aging (Núñez et al. 2018). Importantly, global assessment of aged murine bone masked region-specific deterioration of the cortex highlighting the necessity to assess specific regions of the bone cortex. This ability to regionalise the cortex permits the combination of techniques such as SR CT and finite element analysis (FEA) (Núñez et al. 2018; Zahm et al. 2010) to assess the effects of load (Donaldson et al. 2014; Larrue et al. 2011) and microstructure (van Tol et al. 2020) on cortical bone. However, as this method is developed for the murine TFJ, further validation is required to determine its applicability in investigating regional-specific functions of the bone microstructure at other anatomical sites.

Regionalisation of the TFJ revealed that the posterior TFJ possessed a greater number density of intracortical canals versus the other regions of the TFJ concordant with the literature (Núñez et al. 2018; Maeda et al. 2020). The finding that intracortical canal distribution is heterogeneous is not specific to the murine TFJ: at the murine femoral midshaft, the lateral region possesses more canals than the posterior region (Schneider et al. 2007) while in humans (Perilli et al. 2015) the medial proximal femoral shaft houses more intracortical canals than the anterior and lateral regions. This may suggest the abundance of intracortical vasculature may vary between specific anatomical sites; however, what links these sites and the underpinning vascular heterogeneity remains unspecified. One hypothesis is that mechanical strain may govern the formation of intracortical canals: the inhomogeneous distribution of mechanical strain at the murine TFJ, and the relative absence of mechanical strain in the posterior region (Núñez et al. 2018) could suggest that mechanical strain negatively regulates the formation of the bone vasculature. Alternatively, whether these abundant intracortical canals within the posterior TFJ provide a specific function is unknown. One suggestion is that the posterior TFJ is a region of thicker bone cortex (Núñez et al. 2018), suggesting that such intracortical canals deep within the posterior cortex may prevent formation of a hypoxic microenvironment to sustain populations of lacunae deep within the bone cortex. Here, we report that large osteocyte lacunae are located around this distinct population of abundant posterior intracortical canals, suggesting that this subset of canals may provide a specific, osteogenic function tied to large osteocyte lacunae.

Variability in mean osteocyte lacunae volume has been observed in healthy murine (Núñez et al. 2018) and human bone (Carter et al. 2013), as well as across taxa including ray-finned fishes (Davesne et al. 2020), suggesting that variability in osteocyte lacunae volume is a conserved feature for the maintenance of cortical bone. Further, in diseased human bone, osteopenic bone is characterised by large osteocytes while osteoarthritic bone by small osteocytes (van Hove et al. 2009). The function of osteocyte lacunae heterogeneity – in particular, large lacunae associating with the bone vasculature within in specific bone regions - is unclear, with hypotheses primarily focused on the potential role of the LCN in mechanotransduction (Schurman, Verbruggen, and Alliston 2021; Qing et al. 2012; Lane et al. 2006) and bone (re)modelling (Bonewald 2011; Kennedy et al. 2014).

Alterations to the volume of the LCN has the potential to inhibit bone's mechanoresponse, with increased LCN volume observed in aged mice alongside a reduced LCN connectivity, the latter of which dampens the LCN's mechanostimulatory response. Interestingly, expansion of the

osteocyte lacunae may rescue this mechanosensory deficit, suggestive that enlargement of the pericellular space may be compensatory to respond to a reduced LCN connectivity (Schurman, Verbruggen, and Alliston 2021). While the potential for a heterogeneous distribution of large osteocyte lacunae playing a role in the adaptation to reduced canalicular connectivity requires further interrogation, the reporting of heterogeneity in osteocyte canalicular length and load-induced fluid flow at murine TFJ (van Tol et al. 2020) does suggest that these adaptions to LCN degeneration may be region-specific. This, in tandem to the finding that the posterior TFJ is susceptible to microstructural degeneration (Núñez et al. 2018), may highlight this region particularly prone to degeneration and as such, the presence of large osteocyte lacunae may be indicative of a larger LCN response to microstructural degeneration.

Alternatively, the role of osteocytes in bone (re)modelling (Bonewald 2011; Kennedy et al. 2014) could suggest that the heterogeneous distribution of large osteocyte lacunae facilitates regional bone formation – such as during perilacunar remodelling (PLR). Regulation of osteocyte lacunae and canalicular volume through local matrix resorption/deposition is observed in response to lactation (Qing et al. 2012) and glucocorticoid treatment (Lane et al. 2006). Therefore, the population of large lacunae at the posterior TFJ region may be lacunae currently resorbing the surrounding matrix, increasing their lacunar volume. Should large lacunae differentially affect the composition of the local matrix versus small lacunae in specific regions of bone, their potential for the regulation of bone mineral may provide avenues for the research of region-specific bone anabolic agents.

However, the correlative appearance of abundant intracortical canals and large lacunae may suggest a paired functionality. Assessment of the regional spatial organisation of osteocyte lacunae in relation to intracortical canals in this study found that that posterior intracortical canals prevent the isolation of a subset of large lacunae. This may suggest that preferential vascular support of large lacunae by intracortical canals is a region-dependent phenomenon, which may facilitate a region-specific function. The posterior TFJs susceptibility to microstructural deterioration (Núñez et al. 2018) and heightened remodelling response to loading (van Tol et al. 2020) may highlight the candidacy of the posterior region of the TFJ as a site of unique angio: osteogenic coupling.

Heterogeneity in vascular functionality and the facilitation of osteogenesis is well described (Ramasamy et al. 2014; Kusumbe, Ramasamy, and Adams 2014; Ramasamy et al. 2016), with a specific vascular subtype - type H blood vessels - shown to be a key regulator of osteogenesis.

Type H vessels have been shown to upregulate osteoprogenitor survival and proliferation factors through Notch (Ramasamy et al. 2014) and HIF1α (Kusumbe, Ramasamy, and Adams 2014) signalling. However, how Notch signalling regulates lacunae volume is unknown, with Notch signalling in osteocytes inducing an osteopetrotic (high bone mass) phenotype – a condition associated with small, spherical, non-aligned lacunae (van Hove et al. 2009). Furthering our understanding of how specific vascular subsets regulate osteocyte volume and the regulation of PLR may elucidate how and why these large lacunae exist.

Thereby, we hypothesise that removal of factors coupling type-H vessel-driven angiogenesis and osteogenesis would prevent the formation of large osteocyte lacunae surrounding posterior intracortical canals. Previously, induction of hype-H endothelial cell formation has been shown to elevate bone marrow and serum levels of VEGF (Xie et al. 2014). Furthermore, osteocyte-like cell lines express VEGF in response to shear stress placed on canaliculi (Harper, Gerstenfeld, and Klagsbrun 2001) and hypoxia (Mulcrone et al. 2020). These findings could suggest that in response to mechanical and hypoxic stimuli, osteocytes have the potential to regulate neo-angiogenesis.

As such, the organisation of osteocyte lacunae was assessed in a murine model with conditional VEGF deletion in osteocalcin expressing cells (Goring et al. 2019). Here, we show that at the posterior TFJ of male OcnVEGFKO mice, lacunar volume was unaffected by proximity to the intracortical vasculature. This finding advances our knowledge of the angiogenic, sexually dimorphic effects of VEGF (Goring et al. 2019) and its regulation of the bone microstructure. We have previously shown that male OcnVEGFKO bone is characterised by mineralisation deficits and increased SOST expression (Goring et al. 2019), suggesting that loss of VEGF may affect osteocyte function. Further, previously described disruption of the bone mineral within OcnVEGFKO bone and this study's described inability to form this sub-population of large osteocyte lacunae in the posterior region of the TFJ may suggest that these large osteocytes play a crucial role in the regulation of bone mineralisation. How osteocytes regulate the bone mineral via SOST carries the potential for the expansion of mechanistic context for SOST antibodies including romosozumab (Reviewed (Fabre, Funck-Brentano, and Cohen-Solal 2020); could romosozumab be influencing osteocyte regulation of the bone mineral via PLR, rather than regulation of downstream wnt signalling pathways regulating osteoblasts and osteoclasts?

Moving forward, assessment of the mineral surrounding canal associated osteocytes in health, disease and treatment states could provide insight to potentially unique functionality of these canal associated osteocytes. Currently, techniques that enable the 3D assessment of osteocytes within their lacunae and subsequent relation to the mineral matrix (i.e. without decalcification) are limited, with the majority of applications lacking sufficient soft tissue contrast and resolution to visualise the osteocytes within lacunae.

The identification of a population of large osteocyte lacunae closely associated with densely vascularised cortical regions such as the posterior TFJ, suggests that within specific regions of the bone cortex, there may be regions utilising unique osteo: angiogenic-coupling mechanisms that may underpin the regulation of osteocyte lacunae volume. Furthermore, we show that in the absence of the key angiogenic factor, VEGF, these large osteocyte lacunae do not form at the posterior TFJ, suggestive of uncoupling of this region specific osteo: angiogenic signalling.

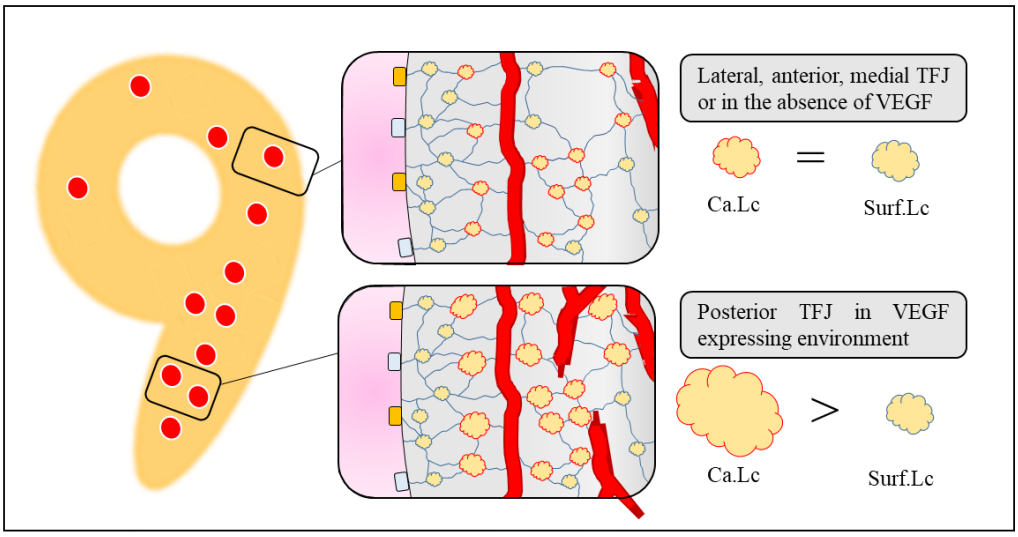

Fig. (7): Canal proximity within the posterior TFJ supports large osteocyte lacunae. WT mice possess larger canal associated lacunae than surface associated lacunae, specific to the posterior TFJ with canal proximity not relating to changes in lacunar volume in anterior, lateral and medial regions. In contrast, in OcnVEGFKO bone canal associated and surface associated osteocyte lacunae at the posterior TFJ are homogenous in volume. Disruption of canal associated lacunar volume by VEGFKO could be indicative of a role of VEGF in the direct regulation of the osteocyte lacunae network and its interactions with the bone vasculature.

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Authors' roles: Study design: JT, AS, PG, CEC. Data collection: JT, AS. Data analysis JT. Data interpretation: JT, CEC. Drafting manuscript: JT, PS, and CEC. JT takes responsibility for the integrity of the data analysis.

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