THE MICROSTRUCTURE OF OSTEOCYTIC LACUNAR-CANALICULAR SYSTEM VARIES WITH AGE AND BONE MINERAL DENSITY IN OSTEOPOROTIC AND OSTEOARTHRITIC PATIENTS

by

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<table>
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<tr>
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<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>AR</td>
<td>Aspect ratio or 2D anisotropy</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Airy units</td>
</tr>
<tr>
<td>BMD</td>
<td>(Material) Bone mineral density</td>
</tr>
<tr>
<td>Circ.</td>
<td>Circularity</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LCS</td>
<td>Lacunary-canalicular system</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NaFl</td>
<td>Sodium fluorescein</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OP</td>
<td>Osteoporosis</td>
</tr>
<tr>
<td>px</td>
<td>Pixel</td>
</tr>
<tr>
<td>TMD</td>
<td>(Apparent) Tissue mineral density</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>µA</td>
<td>Microampere</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>µε</td>
<td>Microstrain</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>VOX</td>
<td>based on counting voxels</td>
</tr>
<tr>
<td>DT</td>
<td>based on distance transformation (filling structure with spheres)</td>
</tr>
<tr>
<td>TRI</td>
<td>based on triangularization of surface</td>
</tr>
<tr>
<td>TV</td>
<td>Total Volume (mm(^3))</td>
</tr>
<tr>
<td>BV</td>
<td>Bone Volume (mm(^3))</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Relative Bone Volume ([1]) (%)</td>
</tr>
<tr>
<td>Conn.D.</td>
<td>Connectivity Density, normed by TV (1/mm(^3))</td>
</tr>
</tbody>
</table>
| SMI     | Structure Model Index:  
0 for parallel plates, 3 for cylindrical rods |
| DT-Tb.N | Trabecular Number (1/mm) |
| DT-Tb.Th| Trabecular Thickness (mm) |
| DT-Tb.Sp| Trabecular Separation = marrow thickness (mm) |
Osteocytes form an interconnecting network in bone and play an important role in maintaining bone health. They reside in ellipsoidal spaces called lacunae and communicate with other cells by extending dendrites known as canaliculi. The osteocyte lacunar-canalicular system (LCS) also functions as a means of transporting fluid, nutrients, and signaling molecules in bones. LCS parameters, such as lacunar density, lacunar size, and the number of canaliculi emanating from individual lacunae, differ in patients and may function as indicators of bone health. While there have been ample studies providing information on the LCS of animals such as mice, quantitative data concerning human LCS is currently lacking.

The osteocyte LCS from 19 osteoporotic and 6 osteoarthritic patients were quantified and compared in this study. The patients included 8 males and 17 females with age range from 51 to 95 years old. Among the osteoporotic group, 10 patients suffered fracture. Transverse sections of cortical bone, from the superior side of the femoral neck, were stained with sodium fluorescein, imaged with confocal laser scanning microscopy, and analyzed for LCS parameters. Micro-computed tomography was used to obtain the cortical bone/tissue mineral density for the same samples.

The results demonstrate that the lacunar perimeter and number of canaliculi significantly increase with age for the osteoporotic group, yet tend to decrease (although not significantly) for the osteoarthritic group. Lacunar size tends to be larger in males but was not statistically different from that in females. Canalicular density (i.e., the number of canaliculi per unit length of lacunar perimeter) does not vary with
age or any patient category (males vs. females, osteoporotic vs. osteoarthritic, fractured vs. non-fractured), in agreement with our previous results in mice. Interestingly, we found that the canalicular density increases with bone mineral density with a very high significance ($R^2 = 0.30$ and $p = 0.005$) mainly due to the decreasing lacunar perimeter with higher bone mineral density. For our knowledge, this is the first experimental evidence that osteocytes actively sense their matrix environment in terms of mineral content and adapt their morphology accordingly.

In summary, the current investigations provided much needed quantitative data on human osteocyte LCS microstructure and its relations to age, gender, and bone mineral density in a mixed patient population with osteoporosis and osteoarthritis. The intriguing finding of increased canalicular number density in denser bone matrix needs to be tested in normal human population in the future. The potential cellular and molecular mechanisms by which osteocytes sense and adapt to their environment in both healthy and disease conditions remain to be determined. This line of research will enhance our understanding of how to maintain the health of bone cells and improve bone quality in humans.
Chapter 1

GENERAL INTRODUCTION

The Osteocyte

The osteocyte is one of three bone cell types found in mature bone tissue and is certainly the most abundant. Osteocytes make up over 90% of all bone cells in an adult skeleton (Bonewald 2007) with more than 40 billion individual cells (Buenzli et al. 2015). Osteocytes outnumber the osteoblasts in a ratio of 10 to 1 (Parfitt 1977). Osteocytes also differentiate themselves from other bone cells by their extremely long lifespan. They are viable for decades in contrast to the weeks and days that osteoblasts and osteoclasts live (Bonewald 2007). Osteocytes are located not on the bone surface like other cells but, distributed within the mineralized matrix making up the bone tissue.

Osteocyte Morphology

Osteocytes are star-shaped cells with lengthy and slender processes which are connected to other osteocytes as well as other cells. Estimates of the range of dendritic processes for a single osteocyte vary from 40-60 processes (Tanaka-Kamioka et al. 1998) to 40-100 processes (Beno et al. 2006). The diameter of the cytoplasmic processes can range from 25 nm to 50 nm (Su et al. 2006). The dimensions of the osteocytes differ but are approximately 10 μm by 20 μm in humans (Mullender et al. 1996). Osteocytes are spaced 20 to 30 μm from each other (Sugawara et al. 2005). The
shape of the osteocyte depends on where and how it was formed. In a typical long bone, ellipsoidal shaped osteocytes can be seen in lamellar bone, with short dendrites reaching towards the mineral side and long dendrites extending towards the vascular side. Instead, globular osteocytes with a symmetric spread of short dendrites can be found in flat bones such as calvaria (Ferretti et al. 1999, Palumbo et al. 2004).

**Lacunar-Canalicular System**

The extensive pore network which houses the osteocytes’ cell bodies and their numerous dendritic cell processes is termed lacunar-canalicular system, LCS. The LCS is the major transport conduit responsible for nutrient supply between osteocytes and vasculature as well as cell-cell signaling among osteocytes and surface cells. The canaliculi flow hypothesis proposes that the extracellular fluid within the LCS serves as a medium to activate osteocytes; the flow of the fluid within the microscopically small channels of canaliculi results in appreciable hydrodynamic forces, thus affecting osteocyte mechanosensing. Evidence suggests that LCS parameters, such as the number of canaliculi, may function as an indicator of bone health (Knothe Tate et al. 2004). Bone pathology can be associated with altered lacuna morphology (van Oers et al. 2015).

**Lacuna Morphology**

The lacunae are voids that house the osteocyte bodies. The orientation of the lacuna tends to be aligned with the surrounding collagen in parallel to the direction of load experienced by bone (van Oers et al. 2015). The dimensions of the lacunae differs depending on various factors; they are approximately 5 μm by 20 μm in mice (Su et al. 2006, Chen et al. 2015). The size and shape of lacunae do not differ for women with
and without osteoporotic fractures. Smaller, rounder lacunae are observed in older populations (Carter et al. 2013). There are conflicting reports on whether the lacunae are larger (Wright et al. 1978) or small (Mullender et al. 1996) for osteoporotic patients. Enlarged lacunae can be observed in lactating, pregnant or calcium-deficient animals (Mullender et al. 1996, Bonewald 2010). In bone tissue, an osteocyte must be situated within a lacuna and each lacuna cannot contain more than one osteocyte; the converse is not true and the fraction of empty lacunae increases with age (Frost 1960). Lacunar density can reflect osteocyte density; this indirect measure is found to decline with age in humans beyond 70 years olds (Carter et al. 2013). Lacunar density decreases with species size for vertebrates (Mullender et al. 1996). Higher lacunar densities are also observed in woven and plexiform bone (Martignetti et al. 2001), mediolateral regions in human femurs (Carter et al. 2014) and those with osteoporosis (Palumbo et al. 1990) or osteogenesis imperfecta (Holmbeck et al. 2005). Despite this, mechanical properties are not affected by osteocyte or lacuna density (Albert et al. 2014). Lacunae only slightly influence the local strain with their size variation (Apostolopoulos et al. 2009).

**Canaliculus Morphology**

Canaliculi are tunnels within an un-mineralized matrix with diameters twice as large as the osteocyte processes they contain. Canaliculi diameter range from 0.5 \( \mu \text{m} \) (Su et al. 2006) to 2.5 \( \mu \text{m} \) (Chen et al. 2015) to around 5 \( \mu \text{m} \) (Ascenzi et al. 2008). Extending outwards from lacuna, canaliculi can contain more than one process and can branch with processes that they contain, and connect to other lacunae or extraosseous spaces. Generally, the canaliculi are not evenly distributed outside a lacuna and the highest density of canaliculi is perpendicular to the bone surface
The number of canaliculi decreases with age (Milovanovic et al. 2013), but the canaliculus density (# of canaliculi per unit perimeter or surface area) appears to be independent of age at least in mice (Lai et al. 2015). Canaliculus density, while not affecting the enhancement of convection, is shown to improve overall transport (Zhou et al. 2009).

**The Origin of Osteocyte**

Osteocytes are derived from osteoblasts, which are derived in turn from mesenchymal stem cells. Osteoblasts are bone-producing cells which largely undergo apoptosis, but can also differentiate into either bone lining cells or osteocytes. It is estimated in humans that 10 to 20% of osteoblasts end up as osteocytes (Liu et al. 1997). There are two differing views on how the osteocyte is embedded in bone matrix. The passive view assumes a process where osteoblasts which becomes osteocytes would reduce their own production rates and eventually become buried by the surrounding osteoblasts (Palumbo et al. 1990, Nefussi et al. 1991, Franz-Odendaal et al. 2006). The active view is that active matrix degradation is required to produce the lacuna and canaliculi (Zhao et al. 2000, Holmbeck et al. 2005). In remodeled cortical bone, the osteocytes form in tunnels caused by bone-removing osteoclasts. Starting from the mineralized tunnel wall, osteoblasts that are polarized towards the mineralized surface become an osteoblastic osteocyte (Nefussi et al. 1991). It then becomes an osteoid osteocyte by first extending short tendrils towards the mineralized surface, before extending long tendrils toward the vasculature at the center of the tunnel, and remain in contact with the osteoblasts (Palumbo et al. 2004). An osteoid osteocyte can also regulate minerals, forming calcified spheres on their processes that initiate mineralization of the matrix (Barragan-Adjemian et al. 2006). Proponents of
the active view suggest that the tendril-extension process is accompanied by the cleaving of collagen (Holmbeck et al. 2005). An embedded osteoblastic osteocyte can mature, turning from an osteoid osteocyte to a young osteocyte. The young osteocyte can in turn mature into an old osteocyte. It is suggested that mineralization can be detected by the osteocyte, which serve as a trigger for maturation (Irie et al. 2008) The cell size decreases while the number of organelles drops. These osteocytes and pre-osteocytes differ from osteoblasts in their gene expression. Osteocyte specific markers include DMP-1, MEPE and PHEX (Dallas et al. 2010). DMP-1 and MEPE may be related to mineralization and demineralization (Gluhak-Heinrich et al. 2007). E11/gp38, which is related to tendril formation and growth (Zhang et al. 2006), is expressed by pre-osteocytes and young osteocytes. Sclerostin, which can inhibit the Wnt signaling pathway (Li et al. 2005, Ellies et al. 2006) and osteoblast bone formation (Bellido et al. 2013, Burgers et al. 2013), is expressed once the osteocyte leaves the transitional stages (Dallas et al. 2010).

**Osteocytes as Mechanosensory Cells**

Mechanical loading influences osteocyte gene expression (Skerry et al. 1989, Gluhak-Heinrich et al. 2003, Gluhak-Heinrich et al. 2007, Robling et al. 2008), indicating its capability as a mechanosensory cell. Round osteocytes are more particularly sensitive (van Oers et al. 2015). It was shown that osteocytes have a detection threshold of approximately 10,000 με (Verbruggen et al. 2015). Although the strain detected in the tissue caps around 3000 με (Schaffler et al. 2014), an explanation suggested for this was that the LCS and its interstitial fluid, can amplify the osteocyte strain above the threshold. There are three types of osteocyte sensing mechanisms proposed (Duffy et al. 2015). The process-adhesion mechanism states
that the flexible proteoglycan which adheres the processes to the canaliculus wall (Schaffler et al. 2014) could produce the amplification with fluid flow. It also suggests that the maximal strain occurs at the processes supports this mechanism (Anderson et al. 2005). The focal adhesion mechanism suggests that stimulus at the cell membrane adhesion can be signaled to the nucleus by a mechanosome (Bidwell et al. 2010), considering that amplified lacuna strain also surpasses the threshold (Nicolella et al. 2006). The direct flow-sensing mechanism states that direct flow-sensing at the cell membrane is possible, it was demonstrated that fluid flow alone can trigger osteocyte response (Qin et al. 2003). The osteocyte primary cilium, which deflects with just 0.03 Pa of shear stress (Malone et al. 2007), is identified to be a promising flow sensor with its sensitivity.

Osteocytes release nitric oxide (NO), prostaglandin and ATP in response to shear stress (Bonewald 2011). NO and prostaglandin promote bone formation. NO also inhibits bone resorption (Forwood 1996, Bakker et al. 2001), thus suggesting osteocytes’ role in bone response to loading.

**Osteocyte Regulation of Osteoblast and Osteoclast Functions**

Osteoblasts and osteoclasts, which carry out bone formation and resorption, are effector cells. They permit both the adaptation of bone to loading and the maintenance and repair of bone. There is evidence that osteocytes, which can communicate with osteoblast via gap junctions (Doty 1981, Marotti et al. 1992) and with both osteoblasts and osteoclasts via extracellular paracrine signaling, are the orchestrators of the functioning of osteoblast and osteoclasts.

Prostaglandins and NO are produced by osteocytes, especially when there is mechanical stimuli (Forwood 1996, Forwood et al. 1998, Bakker et al. 2013). They can have anabolic effects on bone but these are limited in range (Hakim et al. 1996, Mancini et al. 2000, Fortier et al. 2001, Nakalekha et al. 2010), suggesting selective signaling or signal propagation (Schaffler et al. 2014).

Osteocytes are also stated to recruit osteoclasts. Apoptotic osteocytes are observed where the bone resorption rates are high including cases caused by disuse or estrogen deficiency (Noble et al. 1997, Verborgt et al. 2000, Emerton et al. 2010, Cabahug et al. 2013). These apoptotic osteocytes had been engulfed by osteoclasts while under observation (Elmardi et al. 1990, Cerri et al. 2003). While preventing osteocyte apoptosis stops any osteoclast remodeling (Cardoso et al. 2009), mere inhibitory molecules were insufficient in explaining osteoclast recruitment, as few differentiated osteoclasts are normally present (Schaffler et al. 2014). RANKL is the active pro-resorptive signal which triggers osteoclast differentiation; its deletion leads to high BMD (Xiong et al. 2011). When osteocytes undergo apoptosis due to damage
caused to their processes by microcracks, nearby surviving osteocytes were found to express RANKL (Verborgt et al. 2002), which may reach bone surface or vasculature to act on osteoclast precursors (Schaffler et al. 2014).

**Osteocyte Maintenance of Bone Mineral Homeostasis**

The porosity of the osteocyte LCS can reach up to around 15% (Schneider et al. 2007, Schneider et al. 2011, Fan et al. 2016). The total surface area lining the LCS pores is estimated to be far greater than the surface area of endosteum and periosteum (Marotti et al. 1995). With such a large surface, the removal or addition of just thin layer of mineral from the perilacunar matrix could contribute significantly to bone mineral homeostasis. The idea that osteocytes can remove perilacunar matrix is known as “osteocytic osteolysis” (Belanger et al. 1967). It is supported by the unusually large lacunae observed in patients with pathologies such as hemodialyzed uremia (Bonucci et al. 1976), renal osteodystrophy (Bonucci et al. 1977) or Paget’s disease (Bélanger et al. 1968) and patients taking PTH (Mosekilde et al. 1978). This potentially explains the bone calcium regulation not accounted by osteoclastic bone resorption (Ramp et al. 1971). However, the idea was criticized since plastic embedding was suggested to cause artifacts and enlarge lacunae in pathological bone where the larger lacunae were observed (Parfitt 1977). Osteocytic resorption was also calculated to be too weak to guarantee ion homeostasis (Baylink et al. 1971). These pathologies cannot explain the observations of enlargement of lacunae in animals during periods of high calcium demand, such as breeding *Vipera aspis* (Alcobendas et al. 1991), hibernating golden hamsters, hibernating ground squirrels (Haller et al. 1977), lactating brown bears (Kwiecinski et al. 1987), green iguanas on a low calcium diet (Anderson et al. 1976) and sexually mature eels (Lopez et al. 1980). Some of these animal perform no
osteoclastic bone remodeling in the periods where the enlargement of lacunae were observed, strengthening the idea that osteocyte can remodel their surround matrix. The canaliculi around the osteocyte processes were also shown be demineralized in osteopetrotic mice lacking osteoclasts (Nango et al. 2016). Fish with acellular bone on the other hand display difficulties in regulating calcium when levels are depleted (Takagi et al. 1992), suggesting that osteocytes play a significant role in mineral homeostasis. Lacunar area and osteoclast related genes TRAP and cathepsin K, rise during lactation and return to normal during weaning (Qing et al. 2012). Other studies indicate that osteocytes can also form bone (Zambonin et al. 1982, Zambonin et al. 1982), suggesting osteocyte remineralization occurs after a depletion period. To differentiate from changes due to pathology, the term “perilacunar remodeling” was suggested (Dallas et al. 2013).

Osteocyte Regulates Phosphate Homeostasis in an Endocrine Manner

Osteocytes form a highly connective network by interconnecting with other osteocytes as well as surface osteoblasts, the marrow space and the vasculature through the gap junctions on the processes (Doty 1981, Noble 2008). The gap junctions allow molecules smaller than 1 kDa to be exchanged between connected cells (Bennett et al. 1978). Osteocytes also reside within a LCS network within which fluids are circulated within and though which molecules sized below 70kDa also cross into the bloodstream (Knothe Tate et al. 1998, Feng et al. 2006). This connectivity allow osteocytes to both be influenced by hormones and secrete hormones and other substances. Bone fulfill the criteria for endocrine glands with its connectivity and ability to influence distant organs (Dallas et al. 2013).
FGF23 produced by osteocytes is important in maintaining serum phosphate levels. It decreases expression of NaPi-IIa and NaPi-IIc in the kidney and NaPi-IIb in the intestine to reduce phosphate reabsorption (Miyamoto et al. 2005,Gattineni et al. 2009). The kidney influences osteocytes FGF23 expression in turn (Liu et al. 2006) to produce a negative feedback system. Since dietary phosphorous increases FGF23 levels (Burnett et al. 2006), the reduced reabsorption allows excess phosphate to be excreted and removed. FGF23 also signals the parathyroid gland to decrease PTH secretion. PTH is known to increase FGF23 expression. Pathological FGF23 signaling of cardiac muscle can cause left ventricular hypertrophy (Dallas et al. 2013).

DMP1 and PHEX are also expressed by osteocytes and inhibit FGF23 (Martin et al. 2011). MEPE levels correlate with serum phosphate levels independently of FGF23 (Jain et al. 2004). MEPE may also have endocrine functions since it also extends beyond bone.

**Objectives**

Despite the few known correlations of LCS parameters with pathologies, the amount of information on the human LCS in current literature is lacking. Searching on PubMed for “human and osteocyte and (lacuna or canaliculus)” returns only 43 results with less than ten with quantified LCS morphology. The lack of data is even more severe for patients with osteoarthritis and results are conflicting in the case for osteoporosis. In this study we aim to:

1. Address the dearth of information on human LCS morphology
2. Analyze the effects of age, gender and patient diagnostics on LCS parameters
3. Investigate novel relationships between LCS parameters and bone material property
Chapter 2

METHODS

Specimen Collection

**Source of Specimens:** Human femoral heads were extracted from osteoporotic and osteoarthritic patients in the Christiana Health Care System undergoing semiarthroplasty surgery. Specimens without an intact femoral neck were excluded from this study. Institutional IRB approval was obtained.

**Patient Data:** Specimens originated from 25 patients aged from 51 to 95 years old, and were further classified by the gender of the patient (8 male, 17 female), whether the patient had osteoporosis (19 out of 25) or osteoarthritis (6 out of 25) and whether fracture had been recorded (10 out of the 19 with osteoporosis).

![Figure 1](image-url)  
**Figure 1**  Histogram and pie chart showing age distribution and categories of patients
Figure 2  Flowchart of the entire process; one operator performed every step after pre-processing to minimize variation.
**Pre-processing:** The specimens were collected within 48 hours from the time of surgery and were preserved by refrigeration. Using a DREMEL 4000 High Performance Rotary Tool with a Dremel diamond wheel on the femoral neck of each bone. A triangular-shaped or trapezoidal-shaped wedge, which were thicker on the superior side were extracted. Within the next 24 hours, information on the orientation of the sample was retained by inserting a needle in the medial side. The wedges were then put into a labeled container containing 100% ethanol before being transported to our group.

![Figure 3](image3.png)

**Figure 3** Image of the proximal femoral end. A wedge (red lines) was cut from the femoral neck. All measurements were performed on the superior cortex (blue arrow) (Adapted from Weiss, L., Ed., *Cell and Tissue Biology, A Textbook of Histology*, Urban and Schwarzenberg, Baltimore, 1988)

![Figure 4](image4.png)

**Figure 4** Femoral head as received (left), Rotary tool with wheel saw (center), Femoral neck wedge after sawing (right)
Slide Preparation

**Fixation:** The specimens were fixed by replacing the ethanol in its container with Formalin (10% Formaldehyde solution), a tissue fixative, and were left submerged for at least 24 hours. The specimens were refrigerated at around 4 degrees Celsius whenever possible.

**Cutting:** The specimen was clamped in a metal holder in order for it to be processed by a Buehler IsoMet Low Speed Saw. A large slice of the specimen was first cut and discarded from the medial inferior side. This produced a planar surface on the specimen which allowed further cuts to create large and thin slices originating from the medial inferior end of the wedge that can be eventually imaged with additional processing. Several 0.5mm thin slices were removed from each specimen with the saw for redundancy. The remains of the wedge were used for micro-computed tomography.

![Fixated sample](image1.png) ![Buehler IsoMet Low Speed Saw](image2.png)

**Figure 5** Fixated sample (left), Buehler IsoMet Low Speed Saw (right)
**Polishing:** The 0.5mm slices were manually ground and polished down to 100 to 200μm. This was done by attaching sandpaper onto a granite slab and a plastic block and grinding the slice of specimen between the two manually. The sandpaper used started at #600 grit but was replaced by #800 and #1200 grit sandpaper as the grinding progressed to produce a better finish and to better control the final thickness. Powdered bone left on the sandpaper powder was washed away periodically with distilled water. Once the slices appeared translucent when viewed against an intense light source, they were periodically measured using an Inch Dial Comparator Gage to ensure the thickness did not exceed 200 microns.

![Polishing set including sandpaper, granite slab and plastic block (left), Inch Dial Comparator Gage (right)](image)

**Dehydration:** The polished slices were then dehydrated by submergion in ethanol solutions of ascending concentration (70%, 90% and 100%) Each concentration takes 10 minutes of immersion. Care is taken to ensure we from which specimen each slice were taken.
**Staining:** A sodium fluorescein solution was made by dissolving 30mg of NaFl salt in every ml of 100% ethanol. The solution was kept in containers wrapped in opaque aluminum foil to minimize exposure to light. The dehydrated slices were immediately submerged in the NaFl solution, sharing the same container with slices from the same specimen. The dye was allowed to diffuse for at least 24 hours.

![Figure 7 Foil-covered bottle used for staining (left), Mounted slide (right)](image)

**Mounting:** The stained slices were rinsed with 100% ethanol for around 5 seconds to remove excess dye on the surface of the slice. They were then quickly air-dried before being mounted between a glass slide and its cover slip with Permount. The mounted specimens were then air-dried in a fume cupboard for at least 24 hours while covered to minimize light exposure.
Confocal Imaging

**Settings:** For each patient, a single slice of specimen was examined using a Zeiss LSM 510 confocal microscope (Carl Zeiss Microscopy, LLC, Peabody, MA, USA). Utilizing a 488nm laser line from an Argon laser, at strength 5.0 and 790 gain, the pinhole size was 1AU unless the signal was too weak.

**Region of Interest:** The overall region of interest was everything within the field of view when the slide is viewed through a 5x objective. The field could be broadly separated into 3 zones correspondent to the periosteal, mid-cortex, endosteal regions of the specimen’s cortical region. The most promising area in each zone was viewed through a 20x objective, and this area can be further divided into a 3 by 3 block of 9 sectors. When a feature was to be selected, one of the sectors was randomly picked as the starting point to reduce operator bias.

**Lacunae:** For each patient, 3 lacunae were imaged in each of the 3 zones for a total of 9 lacunae. Within the area imaged for each zone, a new sector was randomly picked to view under a 63x/1.40 oil immersion lens used to view the area at 1x digital zoom and to locate the serviceable lacuna closest to the surface. Focusing on the depth where the lacunar perimeter was at its greatest, an image centered on the lacuna was taken at a resolution of 143 μm x 143 μm (512px x 512px) at a scanning speed 18 seconds per frame, and an average of 8 frames per image. Another new sector was picked if no serviceable lacuna were found, this continued until 3 lacunae were imaged in each of the 3 zones.
Figure 8  5x view of the endosteal zone (red box), a section of which was further inspected with a 20x objective (blue box).
Figure 9 20x of view of the selected area within endosteal zone, which was evenly divided into 9 sectors, from which one sector was randomly chosen (section 6 as outlined in the red box in this sample). One lacuna in this sector (indicated by the cross) was imaged using a 63x objective. The image size was indicated with the blue box.
Figure 10 63x view of the studied lacuna, which was the shallowest lacuna showing the largest projection within the randomly chosen location
**Canaliculi:** For each patient, a block of canaliculi were imaged in each of the 3 zones. Within the area imaged for each zone, a sector was randomly picked to view under a 63x/1.40 oil immersion lens used to view the area at 1x zoom and a 24 μm x 24 μm x 11 μm volume filled entirely with canaliculi in the interstitial area without any lacunae was identified. A 30-layer image stack of resolution 256px x 256px were produced at 6x zoom at a scanning speed of 3.9 seconds per frame, and 4 averages per image. Another new sector was picked if no serviceable block of canaliculi-filled volume was found, this continued until a block of canaliculi-filled volume was imaged in each of the 3 zones.

![Image](image.png)

Figure 11  Volume filled entirely with canaliculi found in the interstitial area within the endosteal zone
**Image Analysis**

**Lacuna Analysis:** 2D images of osteocytes were measured in ImageJ (Fiji) by manually contouring the border of each lacuna, which was observed as the outer border of the ‘ring’ of fluorescent dye within the 2-dimensional confocal images. The pixels within the contour defined the 2D area of the lacuna while the pixels forming the contour defined lacunar perimeter. An ellipse which best fit the contour was then used to obtain major and minor axes. Other derived measures include the aspect ratio (AR), defined as the ratio of lacunar major axis length to lacuna minor axis length, and circularity (Circ.), defined by the formula $4\pi \times \frac{\text{Area}}{\text{Perimeter}^2}$. The number of canaliculi roughly on the same plane as the image was manually counted using the point tool.

![Figure 12](image_url)  
*A confocal microscopy image of a lacuna with its canaliculi; the red contour indicates the manually defined lacunar perimeter while the yellow stars indicates manually counted canaliculi; the width of the image is approximately 24μm*
**Canalicular Analysis:** The canalicular length was measured in ImageJ (Fiji), first applying a 3D Gaussian blur (radius: 2px), then using Otsu’s method to determine the thresholds for individual slices in order to segment the image stack into a binary image stack. The binary image stack was then skeletonized three-dimensionally followed by the pruning of any cycles with the shortest branch method. An analysis of the neighbors of each voxel in the modified image stack would discard any ‘bubbles’ not made of canaliculi. Counting the connections of the voxels produced the length of each branch and segment. The total canalicular length was simply the sum of the length of each branch and segment.
Figure 13  Initial canaliculi image stack as seen in ImageJ (left) and Voxx (right)

Figure 14  Blurring of image stack reduced noise to ease segmentation
Figure 15  Segmented binary image stack; the canaliculi can be distinctly observed in 3D view generated by Voxx

Figure 16  Skeletonized image stack, adding each voxel connection to produce the total canalicular length within the block
Micro-Computed Tomography

Mounting: The same 25 wedge-shaped specimens from which the confocal microscopy slides were scanned in a vertical orientation while submerged in Formalin in the smallest holder that was capable of totally containing the wedge. Any needles were removed to prevent star shaped metal defects. Whenever possible, low-density cloth gauze was used to wrap the sample to prevent motion defects from any relative rotation compared to the holder. Excessively large wedges were ground to fit the holder. 4.27mm of each wedge were scanned.

![Wedge wrapped in gauze within the sample holder](image)

Figure 17 Wedge wrapped in gauze within the sample holder

Settings: Scans were made using a SCANCO medical MicroCT 35 with an x-ray tube potential of 55kVp and an x-ray intensity of 145 μA. A 0.5mm aluminum filter was used to correct beam hardening. The field of view per diameter was 37.5mm resulting in each voxel being 18.5 μm large. An integration time of 800ms was used for each frame and each scan took approximately 40 minutes. 1000 projections spanning 180 degrees of rotation were reconstructed with the associated SCANCO uCT V6.1 software into 231 slices (2048 x 2048 pixels each) via filtered back projection algorithm.
**Region of Interest:** For each sample, a cortical RoI was defined by manually contouring the cortical bone precisely in 5 slides and interpolating that into 50 slides. A trabecular RoI from the same image stack was defined by manually contouring the trabecular bone roughly for an irregular anatomic region a few pixels from the cortical bone for 16 slides and interpolating that to 231 slides.

![Image of contouring](image.png)

Figure 18 Contouring of the cortical bone (left) and trabecular bone (right) in one of the key frames; the cortical bone contouring focus on precision while the trabecular bone contouring avoids the cortical bone and sample edge

**Analysis:** The microCT scans were treated with a Gaussian filter to remove noises and the ROIs were then subjected to auto thresholding with the threshold for trabecular bone to be 35% maximal brightness and that for cortical bone to be 38% maximal brightness. Several standard morphological measures of cortical and trabecular bone were reported for the contoured trabecular and cortical ROIs. In addition, bone mineral density was reported in terms of TMD and BMD. TMD measured the averaged density of all voxels including voids within the volume defined by the
contours (or Region of Interest). On contrast, BMD measured only the averaged density of voxels corresponding to mineralized bone (without those of bone marrows and voids). This was done in the segmented ROIs.
Chapter 3
RESULTS

JMP software, Version 13.1.0 for Windows 7 Enterprise x64 software was used to all the statistical analysis and p-values no larger than 0.05 were considered to be significant.

Effect of Zone on LCS Parameters

The effect of bone location (periosteal, mid-cortex, or endosteal zone) on LCS parameters was investigated using one-way ANOVA. No significant difference was discovered among the three zones for any of the parameters. Thus, for any further results, the averaged value from three zones of each patient was used for further analysis.

Effect of Age on the LCS Parameters of all Patients

One-way ANOVA on a linear regression fit on the 25 patients showed that age tended to have a positive correlation (i.e., slope of the regression line is larger than zero) with most LCS parameters related to lacunar size such as lacunar area, lacunar perimeter (Figs. 19,20) and lacunar major axis (not shown). But the relations did not reach statistical significance. The only significant relation uncovered is that the number of canaliculi increased with age (p=0.04, Fig. 21). Other parameters such as canalicular density, total canalicular length, and BMD appeared mostly unaffected by age (Figs. 22-24).
Figure 19  Scatterplot with a linear regression line depicting lacunar area change with age; shaded area represent 95% confidence fit; a positive relation can be observed but is not statistically significant.

Figure 20  Scatterplot with a linear regression line depicting lacunar perimeter change with age; results are similar to those for lacunar area.
Figure 21  Scatterplot with a linear regression line depicting number of canaliculi per lacuna with age; result is statistically significant

Figure 22  Scatterplot with a linear regression line depicting canalicular density with age; the slope of the regression line is fairly flat and suggests that the canalicular density is not influenced by age
Figure 23  Scatterplot with a linear regression line depicting total canalicular length within a specified volume with age; total canalicular length is almost completely independent of age

Figure 24  Scatterplot with a linear regression line depicting cortical material bone mineral density with age; although there is a slightly decreasing trend of BMD with age, the relation is not significant
Effect of Age on the LCS Parameters for each Gender Subgroup

We investigate if gender influences the age effects on the LCS parameters. The linear regressions were calculated for the male and female subgroups separately; the effects of age on the LCS parameters in the male subgroup are similar to those in the overall population with larger and significant effect sizes in some cases (Figs. 25-30). Age continues to show positive effects on LCS parameters related to lacunar size (such as lacunar area, lacunar perimeter and lacunar major axis). The age effects on the lacunar perimeter (Fig. 26) and major axis were statistically significant (p=0.04 and p=0.03, respectively). Similar to the overall patient population, the canalicular numbers of males are also significantly increased with age (p=0.04, Fig. 27). Other parameters such as canalicular number density and total canalicular length appear unaffected by age in the male subgroup (Figs. 28-29) as in our overall population. Please note that the BMD in our male patients (n=8) showed a decreasing trend with age while that in our female patients (n=17) remained unchanged with age (Fig. 30).

In the female subgroup, however, every parameter appears to show a mostly flat regression slope in relation to age (Figs. 25-30). The surprising result may be due to the opposite trends for the OA and OP patients within the female group (see the subsequent section). No parameter in the female subgroup reaches a statistically significant relation with age, including the canalicular number which is showed to be significantly affected by age in both the overall and male populations in this study (Figs. 25-30).
Figure 25  Scatterplot with the linear regression lines depicting lacunar area change with age; the female subgroup seems unaffected by age while the age effect on the male subgroup is larger than on the female subgroup and the overall patient group.

Figure 26  Scatterplot with the linear regression lines depicting lacunar perimeter change with age; the age effect on males reaches significance (p = 0.04, $R^2 = 0.53$) while no effect is found on females.
Figure 27  Scatterplot with the linear regression lines depicting number of canaliculi per lacuna with age; the results are statistically significant for male subgroup

Figure 28  Scatterplot with the linear regression lines depicting the number of canaliculi per unit length of lacunar perimeter with age; the slopes of the regression lines are fairly flat and suggest the canalicular density is not influenced by age regardless of gender
Figure 29  Scatterplot with the linear regression lines depicting the total canalicular length within a specified volume with age; total canalicular length is independent of age regardless of gender and does not differ between males and females by Student t-test.

Figure 30  Scatterplot with the linear regression lines depicting cortical material bone mineral density with age; the decrease in male group is not significant.
Effect of Age on the LCS Parameters for each Patient Diagnostic Subgroup

We also investigated if disease conditions such as osteoporosis, osteoarthritis, fracture influence the age effects on the LCS. By plotting the linear regression line for each patient diagnostic subgroups separately, it can be observed that for the LCS parameters related to the lacunar size (lacunar area or lacunar perimeter) and the number of canaliculi, the trends are different for the OA subgroups and the OP subgroups, with the said parameters decreasing with age for the OA subgroup, and increasing with age for the OP subgroups regardless of whether fracture occurred (Figs. 31-33). These conflicting trends may explain the relatively flat regression slopes found in the female subgroup and the modest strength of the overall age effects (p >= 0.1). Canalicular density and BMD do not show changes with age (Figs. 34, 36). Canalicular length significantly (p=0.02) increases with age for the OA (no fracture) group, while it tends to decrease with age for the OP no fracture group (p=0.08).

Please note that in this analysis the sample size for each subgroup was relatively small (OA(NoFrac) = 6; OP(NoFrac) = 9; OP(Frac) = 10) and the OA patients are younger than the OP patients.
Figure 31  Scatterplot with the linear regression lines depicting lacunar area change with age; the OA and OP group show opposite trends with increasing age

Figure 32  Scatterplot with the linear regression lines depicting lacunar perimeter change with age; results are similar to those for lacunar area
Figure 33 Scatterplot with the linear regression lines depicting number of canaliculi per lacuna with age; results are similar to those for lacunar area or perimeter.

Figure 34 Scatterplot with the linear regression lines depicting number of canaliculi per lacunar perimeter with age; the high p-values suggest the canalicular density is not influenced by age regardless of patient diagnostic.
Figure 35  Scatterplot with the linear regression lines depicting the total canalicular length within a specified volume with age; total canalicular length displays opposite trends with increasing age for the non-fractured OA and OP patients; the effect is statistically significant for OA group.

Figure 36  Scatterplot with the linear regression lines depicting cortical material bone mineral density with age; no significant age effect is found for each subgroup, although the fractured OP group shows an increasing trend (p=0.2)
Comparisons of the LCS Parameters between Males vs. Females, Fractured vs. Non-Fractured, and Osteoarthritic vs. Osteoporotic Patients

To facilitate comparisons with literature data, t-tests were utilized to observe whether any parameter was significantly affected by gender, fracture or OA/OP (Figs. 37 and 38).

The results indicate that, at least for the overall population in our study, there is no significant difference found for the examined LCS parameters between males vs. females, fractured vs. non-fractured, and osteoarthritic vs. osteoporotic patients (Figs. 37, 38). The canalicular number tends to be higher in the fractured group than non-fractured group, although the difference is not significant (p=0.2, Fig. 37). BMD is lower in the OP group than in OA groups as expected, but does not reach significance (p=0.3, Fig. 38).

We also performed comparisons on subgroups combining any of two factors of gender, fracture, and OA/OP. Bone fracture is identified as a factor which causes significant differences for lacunar perimeter and canalicular number within the male subgroup. Similarly, gender is identified to cause significant differences for lacunar area, lacunar perimeter and canalicular number within the fracture subgroup. These comparisons are shown in the appendix table.
Figure 37  Scatterplots showing comparisons of various parameters between males vs. females, fractured vs. non-fractured, and OA vs. OP; male data points in blue and female data points in red; the line segment connects mean values of the pairs under comparison; none of the comparisons is significantly different (p>0.05) by Student t tests.
Figure 38  Scatterplots showing comparisons of various parameters between males vs. females, fractured vs. non-fractured, and OA vs. OP; male data points in blue and female data points in red; the line segment connects mean values of the pairs under comparison; none of the comparisons is significantly different ($p>0.05$) by Student t tests.
The Associations between MicroCT Measurements and LCS Parameters

It can be observed from previous result sections that for both the overall population and any examined subgroups, the linear regression and group comparisons between males vs. females, fractured vs. non-fractured, and osteoarthritic vs. osteoporotic patients failed to discover any significant effect of age or any patient category on canalicular density (Figs. 22,28,34,38). Canalicular density is highly consistent among all groups with mean values around 0.34 to 0.37 per μm and the second lowest overall coefficient of variation (CV) at 11%. For comparison, the CV for the Lacunar Area, Lacunar Perimeter and BMD is 32%, 15% and 4% respectively. Thus, we performed a more comprehensive investigation between the microCT measurements and LCS anatomical measures.

Multivariate correlations (Fig. 39) identify the ‘BMD - Canicular Density’ correlation with its value of +0.5447 as the only ‘Cortical Descriptor – LCS Parameter’ correlation with an absolute value over 0.5. This effect mostly holds to be true even when using two-way ANOVA analysis to consider interactions or when limiting the data to a subgroup.
Figure 39  Multivariate correlations between the cortical bone properties and LCS parameters; BMD - Canalicular density correlation is the only correlation between the two groups with a value above 0.5
Positive Correlation between Canalicular Density and Cortical Bone BMD

As suggested by the multivariate analysis (Fig. 39), BMD is not significantly associated with LCS parameters other than the Canalicular Density as shown in the linear regressions of lacunar area, perimeter, canalicular number, canalicular density, and total canalicular length with BMD (Figs. 40-44). The strength of the positive correlation between Canalicular Density and BMD varies in each subgroup, but the overall trend of positive correlation remains (Figs. 45, 46).

Figure 40  Scatterplot with a linear regression line depicting the correlation between lacunar area and bone mineral density
Figure 41  Scatterplot with a linear regression line depicting some negative correlation between lacunar perimeter and bone mineral density.

Figure 42  Scatterplot with a linear regression line depicting little correlation between canaliculi number and bone mineral density.
Figure 43  Scatterplot with a linear regression line depicting a positive and significant correlation between canalicular density and bone mineral density (p=0.005)

Figure 44  Scatterplot with a linear regression line depicting no correlation between total canalicular length and bone mineral density
Figure 45  Scatterplot with the linear regression lines depicting the positive correlation between canalicular density and BMD remains within each gender subgroup.

Figure 46  Scatterplot with the linear regression line depicting the positive Canalicular Density-BMD correlation remains even when limited to subgroups with OA/OP and Fracture/Non-fracture.
Summary

For the 25 patients analyzed in this study, canalicular density is highly consistent (range of 0.34-0.37 per μm and coefficient of variation of 11%) and does not change with age, gender, and patient diagnostics (OA/OP and fractured/non-fractured). However, we discovered that canalicular density is significantly increased with BMD. Overall, the lacunar size (area, perimeter) tends to increase with age and the canaliculi number shows significantly increase with age (p=0.04, Table 1). No other significance difference in the LCS parameters was found for their regressions with age and comparisons between groups (summarized in Table 1).

Table 1   Table of the overall results

<table>
<thead>
<tr>
<th></th>
<th>Area (μm²)</th>
<th>Perim (μm)</th>
<th>CanaNum.</th>
<th>CanaDens (1/μm)</th>
<th>CanaLen (μm)</th>
<th>BMD (mg/ccm)</th>
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<td>25</td>
<td>25</td>
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<tr>
<td>mean±SD</td>
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<td>36±5.5</td>
<td>12.5±1.6</td>
<td>0.4±0.04</td>
<td>545.3±131.4</td>
<td>937.8±39.4</td>
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<td>(with age) R²</td>
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<td>0.1</td>
<td>0.2</td>
<td>0.7</td>
<td>0</td>
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<td><strong>0.04</strong></td>
<td>0.7</td>
<td>1</td>
<td>0.7</td>
</tr>
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<td>36.5±7.4</td>
<td>12.6±2.1</td>
<td>0.4±0.1</td>
<td>595.6±118.3</td>
<td>944.8±40.7</td>
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<tr>
<td>Female</td>
<td>70.4±22.1</td>
<td>35.8±4.7</td>
<td>12.5±1.4</td>
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<td>521.6±133.9</td>
<td>934.5±39.6</td>
</tr>
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<td>0.8</td>
<td>0.9</td>
<td>1.0</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
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<td>0.4±0.1</td>
<td>507.5±126.1</td>
<td>953.7±54</td>
</tr>
<tr>
<td>OP</td>
<td>70.8±23.5</td>
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<td>12.6±1.7</td>
<td>0.4±0.04</td>
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<tr>
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<td>1.0</td>
<td>4</td>
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<td>13±1.5</td>
<td>0.4±0.08</td>
<td>564.1±99.1</td>
<td>933.6±35.6</td>
</tr>
<tr>
<td>No Frac</td>
<td>71.3±25.3</td>
<td>35.5±5.8</td>
<td>12.1±1.6</td>
<td>0.4±0.07</td>
<td>532.7±151.2</td>
<td>940.6±42.7</td>
</tr>
<tr>
<td>Frac vs NoFrac p-value</td>
<td>1</td>
<td>0.6</td>
<td>0.2</td>
<td>0.6</td>
<td>0</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Chapter 4

DISCUSSION

Does the Lacunar Size vary with Age, Gender, Disease, and Bone Material Properties?

Within our study on the cortical bone from 25 patients, the LCS parameters related to the lacunar size tend to increase with age (Figs. 19,20). Upon further examination the overall effect can be interpreted as the sum of two opposite trends, i.e., decreasing trend for the OA subgroup but increasing trend for the OP subgroup (Figs.31,32). Since we have more OP patients (n=19) than OA patients (n=6), the overall trend was dominated by that of OP patients. Our osteoarthritis group results agrees with a previous mice study (Lai et al. 2015) with a similar indication of an insignificant decrease in lacunar size. Similarly, our female osteoarthritis subgroup results agree with a previous study completed on deceased women (Carter et al. 2013). We were able to observe a drop in lacunar size but were unable to obtain any statistical significance due to our small sample size for OA women (n=3). Our study showed that for males the lacunar size increases with age (p=0.04 for perimeter, Fig.26) and a similar trend for osteoporotic patients (p=0.04, Figs. 31,32), a phenomenon also observed in calcium deficient mice (Sissons et al. 1984, Sissons et al. 1990). However, for osteoporotic patients the direction that the lacunar size would change with age differs from patients in Mullender’s study (Table 2). Mullender did not find an increase of lacunar size with age, but their results were from trans-iliac biopsies from vertebral collapse patients, which might not be comparable to ours from femoral necks. It should also be noted that all the regressions from ours and Mullender’s did not reach statistical significance (Table 2). Although Mullender found the lacunar size of OP patients to be smaller with older age, another study by Wright
showed that the lacunar size was larger in older patients (Wright et al. 1978, Mullender et al. 1996). We did not find any significant difference on lacunar size between OA/OP patients.

Table 2  
Comparison table of human studies on lacunar size over time

<table>
<thead>
<tr>
<th>Study</th>
<th>Our Study</th>
<th>Mullender et al. 1996</th>
</tr>
</thead>
<tbody>
<tr>
<td>location</td>
<td>femoral neck</td>
<td>iliac crest bone</td>
</tr>
<tr>
<td>n</td>
<td>OA 6</td>
<td>OP 19</td>
</tr>
<tr>
<td>type</td>
<td>6 non-frac</td>
<td>9 non-frac</td>
</tr>
<tr>
<td>gender</td>
<td>3 male/3female</td>
<td>3male/6female</td>
</tr>
<tr>
<td>age</td>
<td>62.7±7.8</td>
<td>69±11.7</td>
</tr>
<tr>
<td>regression coef</td>
<td>-0.83</td>
<td>1.39</td>
</tr>
<tr>
<td>p-value</td>
<td>p=0.6</td>
<td>p=0.1</td>
</tr>
</tbody>
</table>

The decrease in lacunar size in the general population has been attributed to preferential infilling (Carter et al. 2013), where the ability of the osteocyte to inhibit mineralization and maintain its lacunar size is believed to be impaired with aging, leading to mineralization of the lacuna walls and smaller lacunae. This is the same concept of micropetrosis, which was first proposed by Frost (Frost 1960) and later confirmed in several studies. For example, a previous study (Busse et al. 2010) demonstrated increased number of lacunae filled with mineralized tissue (19-35 lacunae/mm²) in old bones (>80 years old) than in younger bones (<39 years old, 2-5 lacunae/mm²). However, there are also possible explanations for enlarged lacunae in osteoporotic patients. Biochemical changes in osteoporotic patients indicate an increase in markers for resorption such as pyridinoline cross-links (Seibel et al. 1993)
and Type I collagen telopeptides (Charles et al. 1994). Given that osteocytes are capable of expressing osteoclastic related enzymes such as TRAP, cathepsin K (Bonewald 2011) and MMP (Qing et al. 2012) as shown in the enlarged lacunae in corticoid treated mice (Lane et al. 2006), and in low calcium conditions (Nordin 1997). It is possible that osteoporosis that pathologically activates resorption by osteoclasts also drives the osteocytes to absorb its surroundings, as lower pH values were observed in lacunas in mice of induced severe osteoporosis (Sano et al. 2015). Perhaps the enzymes (TRAP, cathepsin K), MMPs and acids help the removal of collagens and minerals from the surrounding matrix (Lane et al. 2006). This leads to an increase in lacunar size which outweigh any infilling associated with aging. In our patients, this mechanism could underline the observed trend of increasing lacunar size with aging.

**Does the Number of Canaliculi emanating from each Lacuna vary with Age, Gender, Disease, and Bone Material Properties?**

Our results indicates the number of canaliculi per lacuna increases with age (p=0.04, Fig. 21) for our overall population, which appears to contradict previous studies that found decreasing number of processes per osteocyte with age in mice and rats (Okada et al. 2002, Holmbeck et al. 2005), or fewer number of canaliculi per lacuna in the aged human group (Milovanovic et al. 2013). Despite these discrepancies, the canalicular number found in this study (~12-13) was at similar order for aged subjects (15.9) in contrast to young subjects (22.1), both reported by Milovanovic (Table 3). When patients with osteoporosis in our study were excluded, our linear regression revealed a trend with the number of canaliculi per lacuna decreasing as age increases. It should be noted that the results are not statistical
significant (p=0.7, Fig. 33), perhaps due to the low number of osteoarthritic patients (n=6) in our study. Since our study relies on samples coming from a selective group of patients, our results may not be readily extrapolated into general population. That being said, our study strongly suggests that within osteoporotic individuals the canaliculi per lacuna increases with age (p=0.02, see the appendix). Three explanations are possible: (1) osteocytes in older subjects form more processes during formation, (2) osteocytes can add new processes by degrading the matrix (Holmbeck et al. 2005), or (3) osteocytes with more canaliculi are more likely to survive and to be present in older bones. The first explanation is much simpler than the second explanation and does not require degradation of the matrix. The third explanation is supported by the observation that the fraction of empty lacunae increase with age (Frost 1960). Besides being influenced by age and osteoporosis, canalicular number was not sensitive to gender (Fig. 37) and bone mineral density (Fig. 42) in the patients studied herein.

In parallel, the total canalicular length, which was measured far from the osteocyte body (lacuna), reflects the degree that the osteocyte cell processes penetrate the interstitial area and the interconnectivities among osteocytes. The total canalicular length in our patients showed no correlation with age (p=1.0, Fig. 23) and little correlation with gender (Figs. 29, 38) and BMD (Fig. 44). Our measurements of the total canalicular length within the 24x24x11 μm³ volume (0.074 -0.082 μm/μm³, Table 4) agreed well that reported on femurs from 48-56 years old females (Repp et al. 2017). In contrast to the canalicular number, the total canalicular length increased with age in OA patients (p=0.02) but tended to decrease with age in osteoporotic
patients (p=0.08, Fig. 35). This decreased canalicular length with aging in osteoporotic patients was in agreement with the concept of matrix infilling during aging (Carter et al., 2013) and micropetrosis suggested by Frost (Frost 1960). These results suggest that the pathologies such as osteoarthritis and osteoporosis as well as aging might have differential impacts on the LCS anatomy, depending whether the location is adjacent to cell body or along the cell process. Due to the larger plasma contents and cellular organelles located in the cell body, the lacunar morphology including the number of canaliculi emanating from the body, rather than the canaliculi distant from the cell body, is anticipated to be under active control from the cell’s metabolism.

Table 3  Comparison table of human studies on number of canaliculi

<table>
<thead>
<tr>
<th>Study</th>
<th>Our Study</th>
<th>Milovanovic et al. 2013</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>location</td>
<td>femoral neck femoral mid-diaphyseal</td>
</tr>
<tr>
<td></td>
<td>OA (52-70yrs)</td>
<td>OP (51-95yrs) femoral mid-diaphyseal</td>
</tr>
<tr>
<td></td>
<td>n 6</td>
<td>19 9</td>
</tr>
<tr>
<td></td>
<td>type 6 non-fract</td>
<td>9 non-fract (51-86yrs) femoral mid-diaphyseal</td>
</tr>
<tr>
<td></td>
<td>gender 3 male/3 female</td>
<td>3 male/6 female femoral mid-diaphyseal</td>
</tr>
<tr>
<td></td>
<td>age 62.7±7.8</td>
<td>69±11.7 femoral mid-diaphyseal</td>
</tr>
<tr>
<td>#cana</td>
<td>12.2±1.3</td>
<td>12.1±1.9 femoral mid-diaphyseal</td>
</tr>
</tbody>
</table>

Table 4  Comparison table of human studies on total canaliculi length per μm³ of bone tissue

<table>
<thead>
<tr>
<th>Study</th>
<th>Our Study</th>
<th>Repp et al. 2017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>location</td>
<td>femoral neck femoral mid-diaphyseal</td>
</tr>
<tr>
<td></td>
<td>OA (54-65yrs)</td>
<td>OP (51-95yrs) femoral mid-diaphyseal</td>
</tr>
<tr>
<td></td>
<td>gender</td>
<td>Healthy (48-56yrs)</td>
</tr>
<tr>
<td></td>
<td>age</td>
<td>55</td>
</tr>
</tbody>
</table>

|     | 55 |
Does the Canicular Number Density vary with Age, Gender, Disease, and Bone Material Properties?

Canicular number density, defined as the number of canaliculi emanating from individual lacuna normalized with the size of lacuna, is a new parameter introduced by our group. In our previous studies in mice (Lai et al. 2015), we found that this parameter remained highly consistent (0.22/um² lacunar surface) regardless of the bone location (cortical and trabecular bone), the age (15-32 weeks old), and two disease conditions (perlecan deficiency and diabetes). The constant canicular number density was believed to be a key parameter for ensuring topological stability for the osteocyte network, similar to a computer network. In both cases, the number of links (canaliculi) and their individual bandwidths (canicular dimensions) determine the efficiency of communication among the nodes/cells (Lai et al., 2015). In this study, we aimed to examine whether the canicular number density was conserved in human bone. Indeed, we were unable to observe any significant changes in the canicular density regardless of age, gender, and patient diagnostic (OA, OP, and fracture) (Figs. 22, 28, 34). This conclusion remained true after we controlled for both patient diagnostics and age and their interactions up to second order with two way ANOVA. This confirms the results of reserved canicular density found in our previous mice study (Lai et al. 2015) in human bones; the coefficient of variation for the canicular density also showed nearly perfect agreement between the two studies.
However, one of our most surprising results is that we discovered that the cortical bone mineral density significantly affects canalicular density (p=0.005, Fig. 43), despite canalicular density being independent of most factors including age, patient diagnostics and LCS parameters. In contrast, the total canalicular length measured in the interstitial area does not show changes with BMD (Fig. 44). To the best of our knowledge, this discovery is novel. Since the canalicular density is the ratio of the canalicular number over the lacunar size, the observed BMD-dependent increase in the canalicular density appears to be mostly contributed by the decrease in lacunar perimeter as BMD increases, as it has a steeper slope with a much lower p-value (Fig. 41, slope = -0.04, p = 0.2) compared to the slight increase in canaliculi number with increasing BMD (Fig. 21, slope = 0.01, p = 0.5).

We believe this may be caused by a coping mechanism by the osteocyte to survive possible limited nutrition supply when the osteocyte is encased in a highly mineralized environment. Increase in BMD could reduce the permeability of the bone matrix and restrict nutrition transport to the osteocyte from nearby blood supply. In this case, the osteocyte may decrease in size by autophagy in highly mineralized environments (Dallas et al. 2013) to reduce the need for nutrients; this also explains why osteocyte maturation, which is accompanied by size shrinkage and cell process formation, is triggered by mineralization (Irie et al. 2008). As it has been suggested that the osteocytes by themselves excrete mineralization inhibitors to maintain the surrounding cell space (Nampei et al. 2004), a decrease in size for the osteocytes should also decrease the zone influenced by the inhibitors, leading to infilling of the
lacuna. This in turns could increase the canalicular density, which has been shown to improve solute transport through the LCS (Zhou et al. 2009).

Although the observed increased canalicular density could help maintain osteocyte metabolism and agree with its maturation process in relation with matrix mineralization, the cellular mechanisms for such effect remain to be determined. One scenarios is the following: initially the size of osteocyte cell body and the number of cell processes in newly formed bone are so highly conserved that the canalicular density remains constant regardless of age and gender as demonstrated even in the old bones examined in this study. As the physicochemical mineralization process proceeds and bone mineral density increases, the nutrition supply becomes more restricted and osteocyte cell body reduced in size through autophage. In this scenarios, the osteocyte responds to the increase in BMD by passively change its size. In contrary, another possible scenarios is that late osteoblasts and early osteocytes embedded in the unmineralized matrix may actively sense the higher underlying bone mineral density and initiate reduction of cell body size and formation of more cell processes. There is no evidence to support such active sensing. More mechanistic studies are needed to investigate how canalicular density is determined in bone and why it is altered with BMD.

**Effects of LCS on Osteocyte Mechanosensing**

The LCS porosity determines the overall permeability of the bone matrix, which in turn decides the fluid flow magnitude in mechanically loaded bone (Weinbaum et al. 1994). As we shown earlier in mice (Fan et al. 2016), the canalicular porosity constitutes the majority of the entire LCS (87.5%), suggesting that the
changes of canalicular porosity are anticipated to have greater impact on the tissue permeability. In this study on human samples, we did not detect any significant change in the canalicular porosity with age for the overall patients, although the OA (non-fractured) group tended to have increased canalicular porosity and the OP (non-fractured) group tended to have decreased canalicular porosity (Appendix G). However, the changes in lacunae of these two groups were opposite to their canalicular porosity changes: slight decrease in OA (non-fractured) and slight increase in OP (non-fractured) groups. Therefore, the overall LCS porosity and tissue permeability would remain relatively unchanged for the patients studied herein. We thus do not anticipate changes in the fluid flow and the associated hydraulic forces acting on the cell processes, which are believed to be more sensitive to fluid flow than the cell bodies (Burra et al. 2010). Our morphological measurements of the LCS do not suggest significant alteration on the microfluidic environment experienced by osteocytes. However, there is a general belief that cells become less sensitive to mechanical stimulation during aging. This biological change may apply to osteocytes.

**Strengths and Limitations of the Current Study**

This is the first study to link the morphology of the LCS with bone material property. Our study investigated the microstructure of the LCS in details. Various parameters related to lacunar size and shape, as well as canaliculi, were recorded and examined in relation with age, gender, and bone diseases. Most importantly, bone mineral density and other bone descriptors were recorded along with LCS parameters within the same study, allowing us to discover novel relations.
Another strength is that we used varied categories than usual in the study, allowing investigations into the effect of fracture or gender along with osteoarthritis and osteoporosis. With an increase in the number of samples, the strength of the analysis done on each subgroup would allow the discovery of interesting interactions.

The third strength is the fast processing method that we adopted in this study. Plastic embedding is a major contributor to lead time (Mawhinney et al. 1983). By forgoing this step new samples could be rapidly appended to the study if available. The fixation, staining and air drying stages in our study gave us a lead time of 3 days for each patient; a batch of 10 samples would take a day to process. With embedding, the additional dehydration and polymerization would likely require at least 2 weeks, while the amount of material which needs to be cut or polished would also increase. The quality of image produced using our method is well above sufficient for measuring area and counting canaliculi.

Unfortunately, while our study examined a respectful amount of specimens for a human study, they are rather uneven and lacking in the number of male or OA patients. The small sample size (n=25) reduces the power of our statistical tests; it is likely that an increase in sample size would give us more conclusive results. It may also be desirable to obtain a true control group without any bone conditions, which would allow comparisons between healthy subjects and patients.

A second limitation is the 2D nature of our confocal measurements while the LCS is distributed in 3D in bone. Due to the dye penetration and laser scattering issues
in the relatively large unembedded human samples, we were unable to produce high quality 3D image stacks as we obtained for plastic embedded murine bones (Lai et al., 2015). Most of our LCS measurements were derived from two-dimensional confocal images. However, this allowed us more repeated measurements within the same amount of time. As an alternative, multi-photon excitation microscopy should be able to provide better penetration while greatly reducing scattering and photo bleaching. This should produce 3D image stacks for analysis without requiring plastic embedding.

Please note that our microCT measurements were 3D in nature.

**Future Directions**

All the LCS parameters measured in the study were based on dye perfusion into the pores. Although they serve as indirect measures to cell bodies and cell processes, it would have been desirable to directly measure them in future studies in order to better understand the cell functions. Osteocyte size can be obtained if stained with an appropriate dye, which would aid in confirming whether our hypothesis that infilling lacunae are preceded by the shrinkage of osteocytes. Lacunar density could be measured with nano-computed tomography or confocal image stacks. A cortical bone scan with higher resolution may allow us to exclude lacuna and canaliculi from BMD measurements. The improved BMD measure would not be confounded by lacunar size or canalicular density.

Further investigations could be done to reveal the mechanisms by which canalicular density varies with BMD. To test whether osteocytes from patients with varying BMD possess a conserved of cell size and some innate ability in forming cell
process (maturation), we could attempt to harvest osteoblasts from patients with either low or high BMD, then cultivate them in vitro under the same mineral content and mineralization agents in the culture medium. This allow us to determine whether the osteoblast from different BMD backgrounds are destined to generate the same number of dendrites regardless of the mineral environment. We could also cultivate cells from the same source in environments with varying levels of mineralization. Thus examining the hypothesis that current environment alone determines the number of dendrites. We could also attempt to test our hypothesis that limited nutrition causes osteocytes to shrink by controlling the nutrition flow or concentration in vitro. Three dimension cell culture would be a promising method in attempting to mimic bone matrix in vivo.
CONCLUSION

The current investigations, combining confocal laser scanning microscopy and micro computational tomography of 25 human femoral necks, provided much needed quantitative data on human osteocyte LCS microstructure and its relations to age, gender, and disease conditions such as osteoporosis, osteoarthritis, and fracture.

The results demonstrate that the lacunar perimeter and number of canaliculi significantly increase with age for the osteoporotic group, yet tend to decrease (although not significantly) for the osteoarthritic group. Lacunar size tends to be larger in males but was not statistically different from that in females. Canicular density (i.e., the number of canaliculi per unit length of lacunar perimeter) does not vary with age or any patient category (males vs. females, osteoporotic vs. osteoarthritic, fractured vs. non-fractured), in agreement with our previous results in mice. Interestingly, we found that the canicular density increases with bone mineral density with a very high significance ($R^2 = 0.30$ and $p = 0.005$) mainly due to the decreasing lacunar perimeter with higher bone mineral density. For our knowledge, this is the first experimental evidence that osteocytes actively sense their matrix environment in terms of mineral content and adapt their morphology accordingly.

The intriguing finding of increased canicular density in denser bone matrix needs to be tested in normal human population in the future. The potential cellular and molecular mechanisms by which osteocytes sense and adapt to their environment in both healthy and disease conditions remain to be determined. This line of research will
enhance our understanding of how to maintain the health of bone cells and improve bone quality in humans.
REFERENCES


Burgers, T. A. and B. O. Williams (2013). "Regulation of Wnt/beta-catenin signaling within and from osteocytes." Bone 54(2): 244-249.


Appendix A

APPROVAL BY INSTITUTIONAL REVIEW BOARD (IRB)

MEMORANDUM

DATE: April 11, 2013
TO: Mark Eskander, MD
Orthopedics Research
Christiana Hospital

FROM: Sonia Martinez-Colon

RE: CCC# 33053 - The Efficacy of Treatment with the Novel Peptide CK2.3 in Inducing Mineralization in Human Osteoblasts in Relation to the Subjects' Prior Bone Mineral Density: (DDD# 602228)

This is to officially inform you that your protocol was reviewed and determined to be exempt from Institutional Review Board oversight per 45 CFR 46.101(b) (4) by Jerry Castellano, Pharm.D, CIP, Corporate Director of Christiana Care Health System Institutional Review Board, on 04/10/2013.

Please note that if there are any changes to this protocol, such changes may alter the protocol's exemption status. If you need to make any amendments to your study or if you have any questions or concerns, please contact the IRB Office.

Thank you.

This approval verifies that the IRB operates in accordance with applicable ICH, federal, local and institutional regulations, and with all GCP Guidelines that govern institutional IRB operation.
Osteoblast isolation protocol:

Bone fragments are rinsed in PBS, and are then cut into 1" diameter pieces using a dremel. These pieces are thoroughly rinsed in additional PBS in order to remove bone marrow cells. The pieces are then placed in 75cm² flasks and are digested overnight using supplemented DMEM (1% L-Glutamine, 1% penicillin/streptomycin, 10% FBS) and collagenase. The solution is then removed and spun down for 10 minutes at 1000 rpm, and the supernatant is aspirated. The pellet is then suspended in fresh media, and filtered through 40μm nylon mesh filters. The cells are then plated in 24 well plates, and allowed to grow for 2 weeks with no media changes, after which they are treated with either 40nM BMP2, 100nM CK2.3, or no treatment (negative control), and allowed to grow for an additional 5 days. Afterwards, the cells are fixed and stained as appropriate for the experiment in question.
## Appendix B

### DATA TABLES - OVERALL

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>mean</th>
<th>sd</th>
<th>se</th>
<th>R2</th>
<th>p-value</th>
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</tr>
</thead>
<tbody>
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<td>25</td>
<td>71.3629</td>
<td>23.0576</td>
<td>4.61152</td>
<td>0.0517</td>
<td>0.27435</td>
<td>0.39977</td>
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<td>1.10458</td>
<td>0.08897</td>
<td>0.14756</td>
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<td>0.44776</td>
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<td><strong>CanaNum. By age</strong></td>
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<td>937.796</td>
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<tr>
<td><strong>male</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td><strong>Area By age</strong></td>
<td>8</td>
<td>73.3454</td>
<td>26.5421</td>
<td>9.38404</td>
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<td>0.52711</td>
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<td>0.00608</td>
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<td>2.26774</td>
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## Appendix D

### DATA TABLES – OA/OP COMPARISONS

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| Circ. By OA/OP | 0.67004 | 0.02707 | 0.67634 | 0.06693 | 0.87733 |
| AR By OA/OP | 2.43081 | 0.14424 | 2.22127 | 0.43996 | 0.43756 |
| CanaNum. By OA/OP | 12.5556 | 1.45721 | 12.4474 | 1.43045 | 0.90723 |
| CanaDens By OA/OP | 0.33525 | 0.06134 | 0.35925 | 0.02802 | 0.28983 |
| CanaLen By OA/OP | 519.752 | 154.567 | 522.001 | 135.57 | 0.97994 |
| BMD By OA/OP | 953.061 | 69.9987 | 930.511 | 32.8191 | 0.38764 |

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Appendix E

**DATA TABLES – FRACTURE/NON-FRACTURE COMPARISONS**

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<td>0.34431</td>
<td>0.03962</td>
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<tr>
<td>CanaLen By frac</td>
<td>564.136</td>
<td>99.053</td>
<td>449.556</td>
<td>199.556</td>
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<td></td>
<td>Frac mean</td>
<td>Frac SD</td>
<td>NoFrac mean</td>
<td>NoFrac SD</td>
<td>p-value</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------</td>
<td>---------</td>
<td>-------------</td>
<td>-----------</td>
<td>---------</td>
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<tr>
<td>female op</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Area By frac</td>
<td>63.9665</td>
<td>8.92304</td>
<td>73.7818</td>
<td>33.8731</td>
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<tr>
<td>Perim. By frac</td>
<td>34.648</td>
<td>1.43709</td>
<td>35.9421</td>
<td>6.94199</td>
<td>0.61294</td>
</tr>
<tr>
<td>Major By frac</td>
<td>13.0753</td>
<td>0.91717</td>
<td>13.5252</td>
<td>2.36152</td>
<td>0.62846</td>
</tr>
<tr>
<td>Minor By frac</td>
<td>6.20412</td>
<td>0.99406</td>
<td>6.78318</td>
<td>1.94286</td>
<td>0.47859</td>
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<tr>
<td>Circ. By frac</td>
<td>0.66228</td>
<td>0.06515</td>
<td>0.69509</td>
<td>0.07048</td>
<td>0.38528</td>
</tr>
<tr>
<td>AR By frac</td>
<td>2.25554</td>
<td>0.4927</td>
<td>2.17558</td>
<td>0.39879</td>
<td>0.75096</td>
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<tr>
<td>CanaNum. By frac</td>
<td>12.4722</td>
<td>0.96819</td>
<td>12.4144</td>
<td>2.00136</td>
<td>0.9438</td>
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<td>CanaDens By frac</td>
<td>0.3639</td>
<td>0.01918</td>
<td>0.35306</td>
<td>0.03802</td>
<td>0.49592</td>
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<tr>
<td>CanaLen By frac</td>
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<td>111.549</td>
<td>471.997</td>
<td>158.476</td>
<td>0.24696</td>
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<tr>
<td>BMD By frac</td>
<td>933.098</td>
<td>37.2495</td>
<td>927.063</td>
<td>28.8604</td>
<td>0.74812</td>
</tr>
</tbody>
</table>
### Appendix F

**CORRELATION TABLE – BONE/AGE & LCS COMPARISONS**

<table>
<thead>
<tr>
<th>Measure 1</th>
<th>Measure 2</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD Trab (mmHg/cm³)</td>
<td>BMD Trab (mmHg/cm³)</td>
<td>0.04640 -0.2315 -0.273 -0.2756 -0.1724 0.0762 -0.0814 -0.1333 0.2937 0.0646</td>
</tr>
<tr>
<td>TMD Trab (mmHg/cm³)</td>
<td>TMD Trab (mmHg/cm³)</td>
<td>-0.2178 -0.3713 -0.44 -0.2576 -0.1724 0.0411 0.3011 0.0411 0.1019 0.2099</td>
</tr>
<tr>
<td>DT - Tb.Sp (mm)</td>
<td>DT - Tb.Sp (mm)</td>
<td>0.1809 0.5699 0.5567 0.4614 0.1724 0.0608 0.1724 0.0608 0.0762 0.0762</td>
</tr>
<tr>
<td>DT - Tb.Th (mm)</td>
<td>DT - Tb.Th (mm)</td>
<td>-0.1516 -0.0633 -0.1572 -0.0564 -0.2599 -0.0608 -0.2599 -0.0608 0.0762 0.0762</td>
</tr>
<tr>
<td>DT - Tb.N (mm)</td>
<td>DT - Tb.N (mm)</td>
<td>0.1019 0.1724 0.0762 0.0762 0.1019 0.0762 0.0762 0.0762 0.1019 0.0762</td>
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<tr>
<td>TRI - Tb.Th (mm)</td>
<td>TRI - Tb.Th (mm)</td>
<td>-0.5088 -0.2404 -0.2915 -0.2653 -0.1679 -0.0715 -0.1246 -0.0715 0.0762 0.0762</td>
</tr>
<tr>
<td>Conn.Dens. (1/μm³)</td>
<td>Conn.Dens. (1/μm³)</td>
<td>-0.1984 -0.0532 -0.1899 -0.1886 -0.0564 -0.0564 -0.0564 -0.0564 0.0762 0.0762</td>
</tr>
<tr>
<td>VOX-BV/TV</td>
<td>VOX-BV/TV</td>
<td>-0.2522 -0.3421 -0.4141 -0.3969 -0.2382 -0.1065 -0.1773 -0.1065 0.0762 0.0762</td>
</tr>
<tr>
<td>BMD Cort (mmHg/cm³)</td>
<td>BMD Cort (mmHg/cm³)</td>
<td>-0.2382 -0.3421 -0.4141 -0.3969 -0.2382 -0.1065 -0.1773 -0.1065 0.0762 0.0762</td>
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<tr>
<td>TMD Cort (mmHg/cm³)</td>
<td>TMD Cort (mmHg/cm³)</td>
<td>-0.1903 -0.0532 -0.1899 -0.1886 -0.0564 -0.0564 -0.0564 -0.0564 0.0762 0.0762</td>
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<tr>
<td>TRI - Ct.Th</td>
<td>TRI - Ct.Th</td>
<td>0.0532 0.0532 0.0532 0.0532 0.0532 0.0532 0.0532 0.0532 0.0532 0.0532</td>
</tr>
</tbody>
</table>

**Notes:**
- BMD Trab (mmHg/cm³) and BMD Cort (mmHg/cm³) represent bone mineral density (BMD) at trabecular and cortical sites, respectively.
- TMD Trab (mmHg/cm³) and TMD Cort (mmHg/cm³) represent trabecular and cortical sites, respectively.
- DT - Tb.Sp (mm), DT - Tb.Th (mm), and DT - Tb.N (mm) are descriptors of trabecular size and thickness.
- TRI - Tb.Th (mm) and TRI - Ct.Th describe trabecular and cortical thickness, respectively.
- Conn.Dens. (1/μm³) represents connective tissue density.
- VOX-BV/TV is the ratio of volume of bone to total volume.
- BMD Cort (mmHg/cm³) and TMD Cort (mmHg/cm³) are measures of cortical bone density.
- TRI - Ct.Th is a measure of cortical thickness.
- Conn.Dens. (1/μm³) is the amount of connective tissue per unit volume.
- VOX-BV/TV is a measure of bone volume.
- BMD Trab (mmHg/cm³) and TMD Trab (mmHg/cm³) are measures of trabecular bone density.
- DT - Tb.Sp (mm), DT - Tb.Th (mm), and DT - Tb.N (mm) are descriptors of trabecular size and thickness.
- TRI - Tb.Th (mm) and TRI - Ct.Th describe trabecular and cortical thickness, respectively.
- Conn.Dens. (1/μm³) represents connective tissue density.
- VOX-BV/TV is the ratio of volume of bone to total volume.
- BMD Cort (mmHg/cm³) and TMD Cort (mmHg/cm³) are measures of cortical bone density.
- TRI - Ct.Th is a measure of cortical thickness.
- Conn.Dens. (1/μm³) is the amount of connective tissue per unit volume.
- VOX-BV/TV is a measure of bone volume.
- BMD Trab (mmHg/cm³) and TMD Trab (mmHg/cm³) are measures of trabecular bone density.
- DT - Tb.Sp (mm), DT - Tb.Th (mm), and DT - Tb.N (mm) are descriptors of trabecular size and thickness.
- TRI - Tb.Th (mm) and TRI - Ct.Th describe trabecular and cortical thickness, respectively.
- Conn.Dens. (1/μm³) represents connective tissue density.
- VOX-BV/TV is the ratio of volume of bone to total volume.
- BMD Cort (mmHg/cm³) and TMD Cort (mmHg/cm³) are measures of cortical bone density.
- TRI - Ct.Th is a measure of cortical thickness.
- Conn.Dens. (1/μm³) is the amount of connective tissue per unit volume.
- VOX-BV/TV is a measure of bone volume.
Appendix G

CANALICULAR POROSITY AND CANALICULAR DIAMETER

At the request of the committee the canalicular porosity and diameter were also estimated. The procedure was similar to that utilized for calculating the total canalicular length. In ImageJ (Fiji) a 3D Gaussian blur (radius: 2px) was first applied, then Otsu’s method was used to segment the image stack into a binary image stack. The threshold of each slice was separately determined due to the decrease in brightness in deeper slices. The porosity was found by dividing the number of voxels corresponding to canalicular space by the total number of voxels in the image stack. By assuming that the canaliculi does not branch and that they have circular cross sections, the average canalicular diameter can be calculated by computing $2 \times \sqrt{\frac{\text{porosity} \times \text{total volume}}{\text{total canalicular length}}} / \pi$. The non-branching assumption means the result would be underestimations.

T-tests were utilized to observe whether any parameter was significantly affected by gender, fracture or OA/OP (Figs. 47).

For the overall population in our study, there was no significant difference found for the examined canalicular parameters between males vs. females, fractured vs. non-fractured, and osteoarthritic vs. osteoporotic patents (Figs. 47). The canalicular porosity tended to be higher in the male group than female group, although the difference was not significant (p=0.053, Fig. 47).
Figure 47 Scatterplots showing comparisons of various parameters between males vs. females, fractured vs. non-fractured, and OA vs. OP; male data points in blue and female data points in red; the line segment connects mean values of the pairs under comparison; none of the comparisons is significantly different (p>0.05) by Student t tests.
Linear regression with age showed that (1) for the overall group and the female subgroup canalicular porosity and canalicular diameter remain unchanged with age (Fig 48-51); (2) for males both canalicular porosity and canalicular diameter tend to increase with age (Fig 50-51). The canalicular porosity tend to increase with age for the OA subgroup but decrease with age for the OP subgroup (Fig 52). On the other hand the canalicular diameter tend to decrease with age for the OA subgroup but increase with age for the OP subgroup (Fig 53). It should be note that there was an patient with particularly high canalicular porosity and diameter in all three zones.

Linear regression with BMD showed no change of canalicular porosity or diameter with change in BMD (Fig 54-55).
Figure 48  Scatterplot with a linear regression line depicting the correlation between canalicular porosity and age

Figure 49  Scatterplot with a linear regression line depicting the correlation between canalicular diameter and age
Figure 50  Scatterplot with linear regression lines depicting the correlations between canalicular porosity and age for male and female subgroups

Figure 51  Scatterplot with linear regression lines depicting the correlations between canalicular diameter and age for male and female subgroups
Figure 52  Scatterplot with linear regression lines depicting the correlations between canalicular porosity and age for each patient category.

Figure 53  Scatterplot with linear regression lines depicting the correlations between canalicular diameter and age for each patient category.
Figure 54  Scatterplot with a linear regression line depicting the correlation between canalicular porosity and bone mineral density

Figure 55  Scatterplot with a linear regression line depicting the correlation between canalicular diameter and bone mineral density