SHORT-TERM INTERMITTENT PARATHYROID HORMONE 1-34 ADMINISTRATION AND BONE VASCULAR ALTERATIONS IN C57BL/6 MICE

by

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Abstract

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Advancing age is associated with progressive bone loss, declines in vasodilator capacity of blood vessels, vascular rarefaction and bone marrow blood vessel ossification. Intermittent parathyroid hormone (PTH) administration augments bone volume and is an anabolic agent used to treat osteoporosis in a duration-dependent manner. In animal research, at least 15 days of intermittent PTH administration is required to achieve bone accrual. However, physiological stimuli such as intermittent PTH administration effects the bone vascular system more rapidly; i.e., changes in the bone vascular system precede alterations in bone. Intermittent PTH administration also increases vasodilator capacity of bone blood vessels; however, data regarding its influence on bone blood flow and angiogenesis

are variable. Further, intermittent PTH administration relocated bone marrow blood vessels closer to sites of bone formation, presumably directing blood flow to areas of high metabolism. A potential mechanism by which this may occur is via the secretion of matrix metalloproteinase (MMP)-9, which participates in extracellular matrix remodeling, the migration and homing of cells, and angiogenesis. In contrast, intermittent PTH administration may have unaddressed negative consequences. Bone marrow blood vessel ossification is the progressive conversion of blood vessels into bone with advancing age. Given its role in bone formation, intermittent PTH administration may exacerbate this pathology.

Thus, I sought to quantify ossified bone marrow blood vessels, bone vascular density, the distance between trabecular bone surfaces and bone blood vessels, and the role of MMP-9 in relation to age (Mature vs. Middle-Aged) and short-term (5- and 10-days) intermittent PTH 1-34 administration. The animal ages selected in this dissertation coincide with the initiation of bone loss and bone vascular impairment across the life span. Also, the short duration of intermittent PTH 1-34 administration allows for the assessment of vascular changes prior to the changes in bone.

Specific Aim 1 assessed the influence of short-term (5- and 10-days) intermittent PTH 1-34 administration on bone and bone marrow blood vessel ossification in Mature (6-8mon; n=30) and Middle-Aged (10-12mon; n=30) male and female C57BL/6 mice. Bone parameters were unaltered; however, ossified

vessel volume tended (p=0.057) to increase and ossified vessels were 44% thicker ($p<0.05$) in Middle-Aged vs. Mature mice. Additionally, ossified vessels tended (p=0.08) to be 41% thicker following 10 days of PTH treatment.

Specific Aims 2 and 3 assessed the influence of short-term intermittent PTH 1-34 administration on bone microarchitecture and bone static and dynamic properties, bone vascular density, the distance of bone marrow blood vessels (1-29 μm, 30-100μm and 101-200μm in diameter) from trabecular bone, and MMP-9 density, area and localization in relation to trabecular bone and bone marrow blood vessels. Mature (n=60) and Middle-Aged (n=60) male and female C57BL/6 mice received PTH 1-34 or a vehicle for 5- and 10-days consecutive days. The number of small (1-29μm) bone marrow blood vessels was increased ($p<0.05$) by day 10, coinciding with augmented ($p<0.05$) MMP-9 density closest ($p<0.05$) to these smaller (1-29μm) blood vessels. The overall effects of intermittent PTH administration on the bone vascular system are positive. However, the data reveal a troubling tendency in regards to bone marrow blood vessel ossification, which may ultimately impact blood flow delivery to bone with advancing age.

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Chapter 1

Introduction

The vascular system delivers oxygen and transports nutrients and systemic hormones to bone and bone marrow, and removes waste products [1-3]. In addition, bone blood vessels are also essential components of hematopoietic stem cell niches [4, 5], supply precursor cells to site of bone remodeling [6, 7], and are fundamental constituents of bone multicellular units (BMU) [8, 9] and bone remodeling compartments (BRC) [7, 10]. Since bone cannot exist without a vascular supply, the skeletal system is dependent upon blood vessels [11]. For these reasons, the bone vascular system is crucial for bone growth during development, metabolism and homeostasis.

Long bones (e.g., the femur, tibia, etc.) consists of cortical and trabecular bone, and the medullary cavity within the shaft is occupied by bone marrow [12, 13]. Trabecular bone provides strength and structural support for the ends of the long bone [14], whereas cortical bone forms the diaphysis (i.e., the shaft of the long bone) and outer surfaces of the metaphyses and epiphyses (i.e., the ends of the long bone) [15]. The medullary cavity within the shaft consists of bone marrow cells (e.g., stromal and hematopoietic) and a vascular network. The skeleton is a highly dynamic and active tissue that continuously undergoes remodeling so that newly synthesized bone replaces old or damaged bone [15].

Cortical bone remodeling results from the activities of BMU consisting of osteoclasts, osteoblasts, and capillaries [8, 9]. Trabecular bone remodeling results from the activities of BRC, once again comprising of osteoclasts, osteoblasts, and capillaries [7, 10]. Although BMUs and BRCs exist at different bone compartments (i.e., trabecular and cortical), they perform the same function; i.e., the remodeling of bone. Present within every BMU and BRC is a capillary that provides nutrients, oxygen, and precursor cells to the sites of remodeling. Thus, bone remodeling cannot occur without blood vessels and vascular impairment induced by aging, disease and/or treatment play an integral role in bone homeostasis.

Advancing age often results in vascular decline in terms of diminished bone blood flow [16-19] and reduced vasodilator capacity of bone arteries [17]. In fact, age-related declines in bone blood flow and impaired vasodilation are closely associated with reduced bone volume [17] and strength [16]. Additionally, several investigations have reported declines in bone vascular density (i.e., the number of blood vessels) in aged individuals [20] and animals [21, 22].

In addition to alterations in vascular function and density, a novel pathology has been recently discovered that may have a dramatic impact on bone [22]. Bone marrow blood vessels progressively undergo structural changes, resulting in severe calcium and mineral deposition [22]. This pathology has been coined “bone marrow blood vessel ossification”; i.e., the theoretical conversion of blood vessels into bone [22]. Ossification was observed in young and old rats and in elderly patients with

arteriosclerotic vascular disease and peripheral vascular disease with cellulitis [22]. Progressive ossification theoretically leads to “microvascular dead space”; i.e., loss of vasomotor function, a failure to control blood flow, and diminished patency [22]. Thus, bone vascular dysfunction, which includes bone marrow blood vessel ossification, presumably impairs the delivery of oxygen, nutrients, systemic hormones and precursor cells to bone and bone marrow, contributing to bone loss associated with advanced age.

Bone loss associated with advancing age is often treated with parathyroid hormone (PTH), an anabolic agent used to treat osteoporosis [23]. Under *in vivo* physiological conditions, PTH 1-84 is a systemic hormone produced in the parathyroid glands and mobilizes calcium from bone in response to low calcium levels in the blood [24]. Recombinant PTH 1-34 (i.e., Teriparatide) is a N-terminal fragment of PTH 1-84 and the only FDA-approved bone anabolic agent in the United States [25]. When administered intermittently (i.e., one injection per day), PTH regulates bone cellular communication [23] to induce bone formation. The bone anabolic actions of intermittent PTH administration is duration-dependent, therefore at least 15 days of treatment is necessary to detect bone gains in rodent models [31].

In addition to its effects on bone, PTH influences the vascular system. For example, intermittent administration of PTH improved vasodilator capacity in rat aorta [26] and bone blood vessels [27]. In some investigations, PTH augmented

skeletal perfusion [28, 29]. These vascular alterations coincide with enhanced bone volume and bone mineral density [27, 30-32]. Moreover, physiological stimuli related to intermittent PTH administration effects the bone vascular system quicker than bone [28].

In regards to angiogenesis, 14 days of intermittent PTH 1-84 administration improved bone vascular density in the tibia of mice that corresponded with increased bone formation rate, but did not alter trabecular bone volume [28]. On the contrary, bone vascular density was lower in rats treated with intermittent PTH 1-84 for 15 and 30 days [31]. However, in this investigation, PTH had another effect on the bone vascular network. Subsequent to PTH treatment, bone marrow blood vessels (<29μm in diameter) were spatially closer to sites of bone formation (i.e., osteoid seams) [31]. These data are supported by the examination of blood vessels in human bone biopsies, whereby capillary number was augmented next to sites of bone remodeling [33]. Thus, these data highlight the duel efficacy of intermittent PTH administration on both the skeletal and vascular systems.

Intermittent PTH administration may alter bone vascular density and spatially redistribute bone marrow blood vessels by stimulating the secretion of matrix metalloproteinase (MMP)-9 [34]. MMP-9 has pro-angiogenic properties [35, 36] and cellular homing and migration capabilities [37, 38]. Evidence suggested that 5 days of intermittent PTH administration promoted MMP-9 secretion by osteoblasts and osteocytes in the tibial metaphysis of male Sprague-

Dawley rats [34]. Moreover, MMP-9 not only regulates endothelial progenitor cell number and activity, which is essential for angiogenesis [35], but also stimulates migration of endothelial cells [39] and vascular smooth muscle cells [40]. I speculate that PTH-induced secretion of MMP-9 will serve to relocate bone marrow blood vessels toward trabecular bone surfaces. Thus, understanding the potential role(s) of MMP-9 in angiogenesis and bone marrow blood vessel spatial location is warranted.

Finally, although there are many beneficial aspects of intermittent PTH administration on bone and bone blood vessels, given its anabolic nature on bone, this treatment may inadvertently serve to augment the ossification of bone marrow blood vessels. This outcome would have dramatic impacts on the delivery of blood and nutrients to the skeleton, particularly in the elderly population. Taken together, since the vascular system has regulatory oversight on bone homeostasis and accrual, elucidating the vascular alterations that occur with intermittent PTH administration, a treatment for osteoporosis, will shed light on the link between blood vessels and bone.

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Chapter 2

Review of Literature

2.1 Bone Structure

The skeletal system comprises of appendicular and axial bones. Appendicular bones include the long bones (e.g., femur and tibia) and the short, flat, and irregular bones constitute the axial skeleton [1, 2]. Long bones such as the femur and tibia are subjected to mechanical loading [3-5]. Long bones consist of cortical bone at the outermost layer and in the diaphyseal region [6, 7], and trabecular bone lies at the interior of each end of the long bone (i.e., the metaphyseal and epiphyseal regions) [6, 7]. The shaft of the long bone (i.e., the diaphysis) consists of a medullary cavity containing bone marrow surrounded by the cortical shell [7]. Bone marrow is also present in the intertrabecular spaces of the metaphyses and epiphyses [8], and consists of mesenchymal and hematopoietic stem cells and blood vessels.

Trabecular bone is a porous tissue interconnected by rods and plates [2]. Trabecular bone provides strength and structural support for the metaphysis and absorbs stress from loads placed on the articular cartilage [9]. These stresses are passed onto cortical bone in the diaphyseal region [9]. Trabecular bone surfaces that abut against the bone marrow are called the endosteum. The endosteal surface is protected from the bone marrow environment by delicate bone lining cells [6, 10-

12]. Trabecular bone is described by bone microarchitectural properties, which include bone volume-to-total volume ratio, trabecular number, trabecular thickness, and trabecular separation [13].

Cortical bone is dense tissue arranged in structural units called osteons, where blood vessels and nerves traverse a central canal called the Haversian canal [14]. Cortical bone remodeling results from the activities of BMUs within an osteon [14] and, therefore, the structural integrity of cortical bone depends upon activities within the osteon [14]. The outer part of cortical and trabecular bone is covered by a double-layered membrane called the periosteum, which is important for bone growth and fracture healing [2]. Similar to trabecular bone, the surface of cortical bone facing the bone marrow is called the endosteum, which is a site of greater bone remodeling vs. the periosteal layer [3]. Cortical bone is often described by geometrical parameters including cortical bone volume fraction and cortical thickness [15]. Trabecular bone microarchitecture and cortical bone parameters can be analyzed by bone histomorphometry and three-dimensional microcomputed tomography (μCT).

2.2 Bone Histomorphometry and Microcomputed Tomography (μCT)

2.2.1 Bone Histomorphometry

Bone histomorphometry is a histological technique used to obtain two-dimensional (2D) quantitative analyses of bone microarchitecture and bone cellular

activity from undecalcified bone embedded in plastic (e.g., methyl methacrylate) [16] and from decalcified bone embedded in paraffin wax [17]. Bone histomorphometry is a well-established technique for clinical and research purposes and considered the "gold standard" for assessing structural changes in bone as a result of cellular activity [16, 18]. The nomenclature, mathematical derivations, and units for bone histomorphometric parameters have been standardized by the American Society for Bone and Mineral Research (ASBMR) [19]. It should be noted that there are many bone static and dynamic properties outlined by the ASMBR [19]; however, the investigations in this dissertation analyzed and reported those discussed in the succeeding paragraphs.

The structural parameters examined with bone histomorphometry include the bone microarchitecture for trabecular and cortical bone. Trabecular bone microarchitecture consists of the bone volume-to-total volume ratio (BV/TV, %), which is a percentage of the area occupied by trabecular bone per total tissue volume. Trabecular number (Tb.N, /mm^2) is the mean number of trabecular plates and/or rods per unit area of tissue. Trabecular thickness (Tb.Th, μm) is the mean thickness of individual trabeculae and trabecular separation (Tb.Sp, μm) is the mean distance between individual trabeculae [16, 19].

In addition to assessing bone microarchitecture, bone histomorphometry measures the static properties of bone. Although bone is an extremely dynamic tissue, measurements of bone microarchitecture and cellular activity are often

represented as static parameters; i.e., the measurement at the time of tissue collection. Bone static parameters related to bone formation elucidate the amount of newly formed but not yet mineralized bone (i.e., osteoid) and osteoblast activity. The osteoid surface to bone surface ratio (OS/BS, %) describes the percentage of bone surface covered with unmineralized bone (i.e., osteoid) in comparison to total bone volume. Osteoblast surface to bone surface ratio (Ob.S/BS, %) expresses the fraction of the bone surface covered by osteoblasts, and represents the recruitment of osteoblasts to sites of bone remodeling. The bone static parameters related to osteoclast activity indicate bone resorption. Osteoclast surface to bone surface ratio (Oc.S/BS, %) determines the percentage of the bone surface occupied by osteoclasts actively resorbing bone [16, 19].

Besides bone static properties, bone dynamic properties can be analyzed by bone histomorphometry. Bone dynamic properties assess the changes in bone remodeling across time. These measurements are obtained by fluorescently labeling bone with substances such as tetracycline [19] and/or calcein [20]. Fluorescent labeling of bone allows for the calculation of bone turnover kinetics. For example, administration of two separate doses of tetracycline/calcein separated by several days (e.g., 5 days) will adhere to sites of newly forming and calcified bone, labeling the newly formed bone with one or two fluorescent layers. If bone formation is actively occurring during administration of both doses of tetracycline/calcein, a double fluorescent layer is visible on the bone surface [16] and is expressed as a

double-labeled surface per bone surface ratio (dLS/BS, %). A single fluorescent layer is visible on the bone surface when bone formation is actively occurring at either the 1st or 2nd injection of tetracycline/calcein [16], and is expressed as a single-labeled surface per bone surface ratio (sLS/BS, %). Thus, bone dynamic properties can only be analyzed with fluorescence and are used an indicators of bone formation.

The bone dynamic properties are the mineralizing surfaces to bone surface ratio, mineral apposition rate, and bone formation rate. Mineralizing surfaces to bone surface ratio (MS/BS, %) assesses the percent of newly mineralized bone per total bone surface and is derived from the following equation: MS/BS (%) = dLS/BS + ½ sLS/BS. Mineral apposition rate (MAR) evaluates the rate of new bone deposition and mineralization across time. It is calculated as the mean distance (μm) between double labels divided by the time between the two tetracycline/calcein injections. Bone formation rate to bone surface ratio (BFR/BS, $\mu m^3/\mu m^2$/day) represents how much of the bone surface has mineralized during a given time period (e.g., during the 5 days between the two injections), and is the product of MAR and MS/BS [16, 19].

Taken all together, bone histomorphometry is a useful tool for histological evaluation of bone microarchitecture and bone static (i.e., osteoblast and osteoclast activity) and dynamic (i.e, bone formation) properties. Further, it is the "gold standard" for measuring bone static and dynamic properties.

2.2.2 Microcomputed Tomography

Microcomputed tomography (µCT) is a well-developed, efficient and useful tool for examining bone microarchitecture in small animal models [18], from human bone biopsies [21] and for *in vivo* measurements taken at the extremities in clinical populations [22]. Trabecular and cortical bone can be analyzed with µCT and three-dimensional (3D) volumetric analyses of each bone compartment (i.e., trabecular bone, cortical bone and the marrow cavity) can be visualized separately [18].

Trabecular bone microarchitecture consists of the same parameters as bone histomorphometry; i.e., BV/TV, Tb.N, Tb.Th, and Tb.Sp. The two-dimensional (2D) analyses obtained by bone histomorphometry may have some limitations. For example, the 2D nature of histological methods cannot provide information regarding the entire bone sample, as it measures only the bone present within each bone section on the histological slide. [23, 24]. The three-dimensional (3D) volumetric features of µCT allow for measuring the detailed bone microarchitecture within the entire bone sample [24]. Thus, the more accurate quantification generated by µCT in regards to bone microarchitecture overcomes the limitations of 2D analysis [25, 26].

Cortical bone geometrical parameters are typically assessed by 3D volumetric features of µCT at the mid-shaft of the long bones. Cortical volume fraction (Ct.BV/TV, %) is a percentage of cortical bone volume per total tissue volume and

cortical thickness (Ct.Th, μm) is the mean thickness of the cortical bone in a cross-sectional measurement [19].

2.2.3 Comparison of the Two Methods

Both bone histomorphometry and μCT are tools to assess bone microarchitecture and several shared parameters have been validated between the two methods. Previous studies have evaluated the accuracy and reproducibility of μCT in animal models [27-29] and in human bone samples [23, 30, 31] by comparing the data from μCT with data derived from bone histomorphometry. Results demonstrate that bone microarchitecture measured by μCT was highly correlated with bone microarchitecture analyzed by histomorphometry. For example, when 24 calcaneus bones from human cadavers were analyzed by both μCT and bone histomorphometry, significant correlations were observed for the following parameters: BV/TV (r = 0.69, p<0.01), Tb.Sp (r = 0.90, p<0.01), and Tb.N (r = 0.86, p<0.01) [31]. Further, analyses of human transiliac bone biopsy revealed strong correlations between BV/TV analyzed by bone histomorphometry and 3D μCT (r = 0.93, p<0.05) [23], while a slightly lower linear correlation coefficient was observed in Tb.Th between the two methods (r = 0.85, p<0.05) [23]. The slight discrepancy observed between the Tb.Th parameters resulted from the 3D aspects of μCT, which detects the connectivity between individual trabeculae not detectable with histomorphometry [23].

To summarize, the strong correlations between bone histomorphometry and μCT highlight that both methods are effective tools for evaluating bone microarchitectural properties. The use of both methods are common among researchers since μCT provides finer and more accurate depictions of bone microarchitecture and bone histomorphometry is suitable for analyzing cellular activities in relation to formation and resorption.

2.3 The Bone Basic Multicellular Unit (BMU) and Bone Remodeling Compartment (BRC)

Bone remodeling is a lifelong event. In the mature (i.e., fully developed) skeleton, remodeling results from bone resorption by osteoclasts subsequent to bone formation by osteoblasts [32]. Bone remodeling replaces old or damaged bone in efforts to maintain strength and mineral homeostasis throughout the lifespan of the organism [2]. Bone resorption and formation can take place independent from one another, but during bone remodeling, the actions of osteoclasts and osteoblasts are tightly coupled [11, 32], such that resorption always precedes formation. Bone remodeling processes occur at localized sites called bone multicellular units (BMU). BMUs comprise of osteoclasts, osteoblasts, and capillaries [11, 33]. The BMU was originally described for cortical bone remodeling [34] within the osteon; i.e., the basic structural unit of cortical bone [14]. Due to the cylindrical shape of osteons, the BMU forms a tunnel [11, 33]. Osteoclasts form a cutting edge (i.e., a

cone shape) at the tip of the tunnel and dig into cortical bone during resorption. Osteoblasts trail behind the osteoclasts and seal the tunnel with osteoid during bone formation [11, 33]. Present within every BMU is the vascular network, which traverses the middle of the osteon and supports the cellular activities associated with bone remodeling [11, 33].

Trabecular bone remodeling occurs by similar processes as cortical bone remodeling; however, the BMU forms a hemi-tunnel with the bone cells (i.e., osteoclasts and osteoblasts) moving along the trabecular surface at the remodeling site [11]. More recently, the term "bone remodeling compartment" or BRC was assigned to remodeling at trabecular bone surfaces to distinguish this activity from the activities of the BMU in cortical bone [10]. Similar to BMUs, BRCs comprise of osteoclasts, osteoblasts, and blood vessels [10, 12]. Unlike BMUs, one half of the BRC is unoccupied by bone cells (i.e., osteoclasts and osteoblasts) and is separated from the bone marrow compartment by bone-lining cells [10]. The bone-lining cells are believed to be an integral component of the BRC since they provide a protected remodeling site absent the influence of the marrow environment [10-12]. Blood vessels are also an essential component of the BRC since they transports nutrients, oxygen, and the precursor cells for bone remodeling to these localized sites [10, 35]. For example, trabecular bone remodeling was linearly associated with augmented blood flow in venules (25-50 μm in diameter) located at the site of trabecular bone resorption [36]. Thus, the bone vascular network, especially

capillaries as the substance exchangers, are crucial constituents for bone metabolism (remodeling) and homeostasis.

2.4 The Bone Vascular Network

As evidenced in BMUs and BRCs, several studies demonstrated the importance of blood vessels for bone remodeling [10-12, 32, 33, 35] and demonstrated a positive relationship between bone remodeling and vascular function [36-40]. The section of this review highlights the role of the bone vascular network (i.e., morphology and function) in bone metabolism and homeostasis.

2.4.1 Bone Vascular Morphology

The skeleton is a highly vascularized tissue [8]. As an extension of the systemic circulation, the bone vascular network has afferent, exchange (e.g., capillaries and sinusoids), and efferent vessels [8, 41]. In long bones, the principal nutrient artery (PNA) supplies blood flow to the marrow space of the shaft and the inner 2/3rd of the diaphyseal cortex [8, 41]. The rest of blood flow to long bones is delivered through the metaphyseal, epiphyseal, and periosteal arteries, which supply blood to the region of the long bone where their names were derived. Blood exits the long bones through the efferent veins (i.e., the epiphyseal, metaphyseal, periosteal, and principal nutrient) [8].

The PNA penetrates the diaphyseal cortical shaft, travels to the middle of the marrow space, branches into an ascending and descending limb and navigates parallel to the longitudinal axis of the shaft [8, 42, 43]. Branches of the PNA spread throughout the marrow space and towards the endosteal surfaces, eventually penetrating into the epiphyseal, metaphyseal and cortical compartments [8]. Angiograms of barium sulfate perfused femora revealed that the vascular system in bone is connected throughout the cortex (i.e., cortical bone), periosteum, and medullary cavity [44]. The smaller blood vessels (e.g., capillaries and sinusoid) branch out from afferent arterioles, serving as ionic exchange vessels for bone and bone marrow cells [8, 41, 43]. Capillaries not only support the nutritional and oxygen needs of the tissue, but allow for the exudation of pre-osteoblasts and pre-osteoclasts at sites of bone remodeling [10-12]. Sinusoids are highly permeable capillaries with open pores, a discontinuous endothelial cell layer, and an incomplete basement membrane [43]. The sinusoids perform similar activities as capillaries, but also play a pivotal role in the maintenance of hematopoietic stem cells [45-47].

Figure 2.1. Visualization of bone vascular network by an epoxy resin perfusion.

The efferent vessels drain the blood through the venous system (i.e., from venules to the nutrient veins) and link with the systemic circulation outside of bone [8, 41, 43]. Figure 2.1 shows a representative image of the vascular

network within a rat femur. A red epoxy resin was perfused into the vascular system post-mortem, and the bone tissue was subsequently cleared for visualization of the blood vessels.

2.4.2 Bone Vascular Function

Besides the morphological properties of the bone vascular network, vascular function regulates blood flow and perfusion to bone, and controls the rate and volume of substance exchange. The regulation of bone blood flow occurs via the vasomotor activity (i.e., vasodilation and vasoconstriction) of bone arteries and arterioles, and via vascular density (i.e., the number of blood vessels) within bone and bone marrow. Early researchers developed techniques to assess bone blood flow [48-51]. For example, the ^{18}F radiotracers infusion technique is one means to measure bone blood flow in humans [49]. Data from healthy human volunteers estimated that total skeletal blood flow was ~5% to ~7% of resting cardiac output [49]. In addition, the radio-labeled microspheres technique is the "gold standard" for measuring blood flow in animal models. In a canine model, total skeletal blood flow represented ~11% of cardiac output [52]. These data demonstrate that the skeleton receives a considerable percentage of cardiac output and receives volumes of blood comparable to some skeletal muscles at rest [42, 53].

The volume of blood flow received by the skeleton is site dependent. For example, trabecular bone within the sternum, ribs, ilium, and femoral epiphysis had

greater blood flow rates (i.e., 16-88 $ml \cdot min^{-1} \cdot 100\ g^{-1}$) compared to cortical bone in the femoral and humeral diaphyses (i.e., 0.2-6.0 $ml \cdot min^{-1} \cdot 100\ g^{-1}$) [52]. Moreover, delivery of blood flow is higher to red (i.e., hematopoietic) vs. yellow (i.e., fatty) bone marrow, as demonstrated in a canine model [52]. Similarly, discrepancies in blood flow to various regions of bone were observed in rabbit [51] and rat [54-56] models. For example, femoral bone blood flow in male Fischer-344 rats was highest in the diaphyseal marrow and lowest in the diaphyseal cortex (i.e., cortical bone) [55].

The deviating supply of blood to bone results from the individual characteristics of the bone compartments (i.e., trabecular bone, cortical bone and bone marrow). Diverse oxygen concentrations (pO_2) resulting from metabolic activity and cellularity have been documented. For example, despite the high vascularity of bone marrow, the pO_2 (11.7 mmHg) in this compartment was significantly lower in comparison to the pO_2 (31.7 mmHg) in cortical bone [57], no doubt reflective of the high metabolic activity of bone marrow cells. These data coincide with reports of higher blood flow to areas of bone with high hematopoietic activity (e.g., trabecular bone and bone marrow) vs. areas of low or no hematopoietic activity (e.g., cortical bone) [52, 55]. This concept is further supported by data revealing that hematopoietic marrow is perfused to a greater extent than non-hematopoietic, fatty marrow [52, 55, 58]. Taken together, divergent rates of blood to the different bone compartments are reflective of metabolic rate.

In summary, bone vascular morphology and function are essential to bone and responsible for adequate blood supply in response to metabolism. Capillaries and sinusoids allow for nutrient exchange between blood vessels and bone compartments [8, 41, 43]. Therefore, the alterations in bone vascular morphology and function with aging and disease presumably influences overall skeletal health.

2.5 Bone

During growth and development of an organism, bone formation occurs more frequently than bone resorption. Following skeletal maturity, bone formation and bone resorption are balanced so that bone maintains its mass and density. With advancing age, bone resorption exceeds bone formation, resulting in lower bone mass and density. As observed in rodent models, rapid bone growth occurs during the first six months after birth [59] with the closure of the growth plate in the phalanx, radius, metacarpal, fibula, and tibia occurring at four months of age [60]. Longitudinal growth of long bones occurs at the growth plate [61] and closure of the plate terminates elongation and indicates skeletal maturity [62]. Therefore, rodents >4-6 months old are considered skeletally matured animals. In this section of the review will discuss age-related bone loss following skeletal maturity.

2.5.1 Age-Related Morphological Changes in Bone

Trabecular and cortical bone volume diminishes as a function of advancing age, such that trabecular connectivity is reduced and cortical porosity is increased [63]. The age-related structural changes can be explained by a weakened bone microarchitecture (e.g., reduced Tb.Th and Tb.N and increased Tb.Sp) [64-66]. Weinstein and Hutson (1987) demonstrated that 80 year-old subjects presented with lower (~68%) trabecular bone area in the ilium, resulting from an ~23% ($p < 0.001$) decline in Tb.Th and an ~48% greater Tb.Sp in comparison to 20 year-old subjects ($r = 0.599$, $p < 0.001$). Further, age-related declines in bone microarchitecture were documented in animal models. For example, trabecular BV/TV in the proximal tibia of 20-month-old male and female BALB/c mice was 63% and 54% lower, respectively, than values observed in the 4-month-old counterparts [66]. These declines in BV/TV resulted from a reduction in Tb.N ($r^2 = 0.55$, $p < 0.001$) [66]. Similarly, declines in BV/TV were observed in 22-24 month-old vs. 4-6 month-old male Fischer-344 rats, resulting in higher Tb.Sp [67] and reduced Tb.N [54]. Declines in bone microarchitecture (e.g., BV/TV, Tb.Th, Tb.N and Tb.Sp) result from excessive osteoclastic bone resorption in relation to diminished osteoblastic bone formation [63]. These imbalances in remodeling lead to microarchitectural and structural changes in both trabecular and cortical bone, eventually diminishing bone mass.

Advancing age correlates with imbalances in osteoblast and osteoclast activity, leading to bone loss [64, 68]. For example, serum biomarkers representative of osteoblast (i.e., osteocalcin and N-terminal propeptide of type 1 procollagen [P1NP]) and osteoclast (i.e., Tartrate-Resistant Acid Phosphatase 5b) activity were lower in 12-month-old vs. 6-month-old mice and 23-months-old vs. 5 month-old rats [64, 68]. The reductions in osteoblast and osteoclast activity corresponded with diminished trabecular BV/TV and a reduction in the circumference of L5 vertebrae in the older mice [64], and reduced BV/TV (75%), Tb.N (50%) and increased Tb.Sp (74%) in the proximal tibia of the older rats [68]. Taken together, aging-mediated morphological alterations occur throughout the skeleton, as evidence by alterations in bone microarchitecture and bone cellular activities.

Although less impacted, age-related changes in cortical bone have been documented in the form of diminished bone mineral density (BMD) [69, 70], reduced cortical thickness, increased cortical porosity, and enhanced endocortical diameter [69]. For example, cortical BMD in vertebrae was reduced by 23-30% in women 70 years of age vs. women 30-40 years of age [70]. In the same investigation, the reduction in cortical BMD was lower (11%) but still present in 70-year-old vs. 30-40-year-old men [70]. Cortical bone becomes thinner in advanced age, resulting from the expansion of the bone marrow cavity and from higher erosion rates at the endosteal surface [63, 71]. For example, cortical bone

area was augmented by 34% but cortical bone thickness was diminished by 38% in 24-month-old vs. 3-month-old C57BL/6 mice [71].

Thus, loss of trabecular and cortical bone with advancing age has been demonstrated in the clinical population as well as in rodent models. The deterioration of bone mass via imbalances in osteoclast and osteoblast activity with advancing age predisposes individuals to bone frailty and increased risk of fracture.

2.5.2 Age-Related Functional Changes in Bone

The skeletal system plays a pivotal role in mechanical function, allowing for movement and load-bearing of the body [72]. These functions are impaired with aging, lead to bone fracture and loss of posture [73-75]. For example, age-dependent declines in mechanical function are associated with the loss of bone microarchitecture. Individuals >80 years of age demonstrated 4-10 times higher risk of fracture in comparison to individuals 50 and 60 years of age, despite having similar BMDs [76, 77]. In addition, as a result of diminished bone remodeling [64, 68], bone healing following fracture is delayed in advance age [78], further exacerbating fragility and poor locomotion [79]. A large population-based cohort study from 1991 to 2013 demonstrated that ~151 per 10,000 individuals are hospitalized due to fracture, with higher risks of fracture occurring in the elderly population (> 60 years old) vs. teenagers (12-19 years old) and young adults (20-42 years old) [75]. Similarly, an epidemiological study demonstrated that the annual

incidence of hip fracture in women and men 35 years of age was 3 and 2 per 100,000 individuals, respectively; however this number was exponentially elevated to 2542 and 1893 per 100,000 individuals for 85-year-old women and men, respectively [74].

These age-associated changes have been documented in animal models as well. For example, reduced bone healing in the tibial diaphysis, as evidenced by lower BMD and bone mineral content up to 20 and 25 days and lower bone volume up to 20 days were observed following fracture in 25-month-old vs. 5-month-old mice, resulting in diminished bone strength, poor locomotion and balance [78]. In fact, 45% of the population whom have suffered a fracture have poor balance vs. those without a history of fracture [73]. The inability to maintain position, balance, and movement in old age is reflective of bone function and may further augment morbidity and mortality related to bone disease in the aged population.

In totality, aged-related declines in bone microarchitecture and balance predisposes individuals to higher rates of fracture. Following fracture, the ability to heal is impaired in advanced age, exacerbating bone loss and strength and further augmenting the risk of fracture. This downward spiral may eventually lead to additional morbidity and ultimately mortality.

2.6 Age-Related Alterations in Bone Blood Vessels

Age-related changes in the structural and functional properties of the vascular system include intimal and medial thickening, increased arterial stiffness, and reduced distensibility of arteries [80]. Further, vascular rarefaction (i.e., a reduction in the number of blood vessels) occurs with advancing age, coinciding with impaired microvascular branching and a disorganized geometry within arteriolar and capillary beds [81]. Age-related alterations in vascular function are observed as well, leading to impaired blood flow and perfusion of various tissues. This section describes age-related alterations in bone vascular function and vascular density and describes a newly discovered bone vascular pathology that may have dramatic impacts on bone with old age.

2.6.1 Reduced Vascular Function

Increased vasoconstriction and impaired vasodilation are closely associated with reduced tissue blood flow in old age, leading to a variety of negative health outcomes [82-84]. For example, estimated leg oxygen consumption was diminished by 15%, femoral arterial blood flow by 26%, and vascular conductance by 32% in 63 vs. 28 year-old individuals. In regards to bone, age-related declines in nitric oxide (NO)-mediated, endothelium dependent vasodilation of the femoral PNA [40, 54, 55, 67, 85] corresponded with diminished bone blood flow [54, 55, 86-88], bone microarchitecture (e.g., BV/TV) [54, 55] and BMD [86]. Age-related

decrements in bone blood flow correlated with a 27% reduction in endothelium-dependent vasodilation in the femoral PNA in 24-26 month-old rats when compared to the 4-6 months-old animals [55].

Age-associated decrements in bone blood flow are consistent throughout the skeleton to include the flat (e.g., scapula) and long (e.g., femur) bones; i.e., reduced tibial (-91% and -88%, respectively) and parietal (-87% and -77%, respectively) blood flows were demonstrated in old male and female rats [87]. Age-related decrements in blood flow to the flat and long bones also corresponded with higher vascular resistances, loss of bone material properties, and reduced bone strength [86]. Induction of osteoporosis also leads to diminished bone blood flow; whereby 8 weeks of ovariectomy, which also induced osteoporosis in the 6 month-old female rats, diminished vertebral perfusion vs. sham-operated animals. [89].

Data from animal studies are consistent with those from human subject research. For example, magnetic resonance imaging revealed diminished femoral bone marrow perfusion in elderly (75 year-old) patients with osteoporosis vs. age-matched subjects with normal BMD [90]. Furthermore, in comparison to younger populations, elderly osteoporotic women had diminished perfusion in the bone marrow of the proximal femur, and these elderly women experienced declines in BMD across the 4 year study [91]. Moreover, perfusion to the L3-L4 vertebrae, as measured by computed tomography, demonstrated peak perfusion at 25 years of age, followed by a plateau at 35 years of age, and then a continual decline with

advancing aging, resulting in the lowest perfusion (-41% from the peak value) at 76 years of age or older [92].

Taken together, the changes in bone perfusion with aging in animal models and human subjects are explained by bone vascular dysfunction to include impaired vasomotor activity and blood flow regulation. Age-related declines in angiogenesis are discussed in the following section.

2.6.2 Reduced Vascular Density

Skeletal blood flow is regulated by the vasomotor activities of arteries and arterioles; however, declines in bone blood flow may also result from vascular rarefaction (i.e., loss of blood vessels) or an inability to create new blood vessels via angiogenesis. Vascular rarefaction has been documented in old age [87, 93-97]. For example, an influential study in the 1980s demonstrated reduced vascular density in humans [93]. Utilizing bone histomorphometry on iliac crest biopsies, individuals 30-50 years-old had a lower number of bone marrow arterial capillaries (-49%) and sinusoids (-12%) in comparison to individuals 10-20 years-old [93]. Sinusoidal density further declined by 26% in individuals >70 vs. 30-50 years of age [93]. Interestingly, trabecular bone volume declined along this aging transition and as the number of bone marrow blood vessels were reduced, the volume of marrow occupied by adipocytes was enhanced [93]. Thus, age-related declines in

bone vascular density corresponds with reduced bone volume and augmented fat content in the marrow.

The processes of angiogenesis create new blood vessels [94] and NO supports angiogenesis by stimulating various growth factors [98]. Nitric oxide is produced by endothelial nitric oxide synthase (eNOS) from endothelial cells [98] and the production of NO is suppressed in old blood vessels. For example, NO and eNOS in gastrocnemius muscles were lower in senescence, accompanying a 75% reduction in vascular density vs. young controls [97]. These lower levels of NO in advanced age led to an attenuated (37%) recovery in hindlimb blood flow following femoral artery ligation, while vascular density remained suppressed [97]. In bone, a 25% reduction in endothelium-dependent vasodilation of the femoral PNA in old rats, which coincided with 21%, 28% and 45% lower blood flows to the proximal metaphysis, distal metaphysis and diaphyseal marrow, respectively, resulted from a 70% reduction in NO bioavailability [55]. Furthermore, a new endothelial cell type (Type H) discovered in the bone marrow and tibial metaphysis releases osteogenic factors that aid in bone formation [88, 99]. However, this novel endothelial cell type was diminished in old mice as a result of vascular rarefaction [88, 99], such that tibial bone marrow arteries were reduced by 81% in old (65-70 weeks-old) vs. young (4 weeks-old) mice [99]. Collectively, bone vascular density and angiogenesis are reduced in old age, possibly resulting from impaired NO production.

Taken together, age-related declines in bone vascular density contribute to diminished bone blood flow. Therefore, treatment to alleviate these decrements may aid in maintaining bone blood flow in old age.

2.6.3 Bone Marrow Blood Vessels Ossification

We have been discussing the importance of the bone vascular network for bone. Thus, dysfunction of blood vessels in bone contribute to developing bone-related pathology. To date, little is understood concerning the age-associated structural changes of bone blood vessels. For example, vascular calcification is a well-known pathology associated with aging [100, 101] and certain disease states such as chronic kidney disease (CKD) [102-105], diabetes [101, 106], and atherosclerosis [101, 107]. The underlying mechanisms of vascular calcification are complex and not yet entirely elucidated [103, 108]. Blood vessels have mineral deposition and calcium-phosphate hydroxyapatite in the intimal and medial layers, displaying mechanistic processes similar to osteogenesis [103, 107, 109, 110]. Even though the mechanisms of calcification are similar between blood vessels and bone, a study utilizing solid-state nuclear magnetic resonance (SSNMR) spectroscopy demonstrated discrepancies in the composition of vascular calcification between arterial beds and discrepancies in composition from bone [111]. For example, intimal calcification in human carotid arteries consisted of cholesterol-like complexes, fatty acids, and hydroxyapatite-like carbonate, while medial

calcification of human femoral arteries consisted of fatty acids. Further, SSNMR spectroscopy also revealed a differing composition between intimal calcification of human blood vessels in comparison to tibial cortical bone samples from equines [111].

Vascular calcification has been demonstrated in the bone vascular system as well. Arteriosclerotic lesions were present in arteries in the human femur, with disease progression being advanced by 10 years vs. lesions observed in similar arteries outside of the skeleton [112]. In addition, fibro-fatty intimal plaque and replacement of smooth muscle fibers with collagen was induced in bone arteries of the maxilla and mandible following 20 weeks of an atherogenic diet in rhesus monkeys [113] and evidence of vascular calcification within the adventitial layer of bone blood vessels was reported [114].

To further support the concept of divergent calcification processes between blood vessels from different vascular beds, we recently demonstrated severe calcification and mineralization of the bone marrow vasculature [95], which appears more exacerbated than previous reports of calcification of bone blood vessels [112-114]. This is a novel vascular pathology coined “bone marrow blood vessel ossification” and presumes the conversion of bone marrow blood vessels into bone. To support this theory, Fourier-Transform Infra-Red Spectroscopy (FTIR) performed on bone marrow blood vessels and cortical bone samples from young (4-6 months) and old (22-24 months) male Fischer-344 rats revealed similar FTIR

spectra between old bone marrow blood vessels and old cortical bone (unpublished observations, Prisby 2014). The similarities between old bone marrow blood vessels and old cortical bone occurred in areas of the spectrum that are representative of bone; i.e., the mineral-to-matrix ratio, the carbonate-to-phosphate area, and the crystallinity peak area (unpublished observations, Prisby 2014). This suggests that old bone marrow blood vessels are acquiring the chemical composition of bone. In addition, light microscopic examination of ossified bone marrow blood vessels (Figure 2.2) revealed the presence of osteocyte lacunae (i.e., another characteristics of bone; black arrow) on the abluminal surface [95]. Histological analyses of ossified bone marrow blood vessels in young (4-6 months) and old (22-24 months) male Fischer-344 rats revealed the presence of bone (i.e., ossification) and extensive calcium deposition [95]. The pathology progressed with advancing age such that ossified and calcified vessel volumes increased by 366% and 266%, respectively; (p<0.05) in comparison to the young rats [95]. Coinciding with these alterations, the number of patent bone marrow blood vessels were reduced by 38% [95].

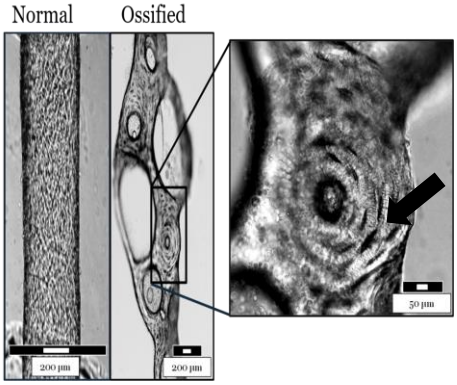

Figure 2.2. Normal and ossified bone marrow blood vessels.

Ossified bone marrow blood vessels represent "microvascular dead space" in bone; i.e., the loss of normal vasomotor capacity (i.e., vasodilation and

vasoconstriction) and patency, and an impaired ability to regulate bone blood flow [95]. This pathology was also present in amputated long bones from elderly individuals with arteriosclerotic vascular disease and peripheral vascular with cellulitis [95], indicating an unaddressed clinical pathology.

In total, calcification and mineralization of bone marrow blood vessels are exacerbated by the aging process. This pathology no doubt contributes to age-related declines in vascular density and blood flow, potentially impacting every bone compartment (i.e., trabecular and cortical bone and bone marrow) in terms of metabolism and homeostasis.

2.7 Effects of Parathyroid Hormone on Bone

Parathyroid hormone (PTH) 1-84 is produced by parathyroid glands and released into the systemic circulation. The major role of PTH 1-84 is the regulation of blood calcium levels. When calcium levels are low in the bloodstream, PTH 1-84 is secreted and stimulates bone resorption to release calcium from bone. When blood calcium levels return to normal, the release of PTH 1-84 from the parathyroid glands is terminated. Thus, the physiological consequences of continuous or prolonged PTH 1-84 secretion is bone resorption [115, 116] as what occurs in pathological conditions such as hyperparathyroidism [117]. On the other hand, when administered as recombinant PTH 1-34 in an intermittent fashion (e.g., an injection per day), bone anabolism is the outcome [118-124]. This section will

review the effects of continuous and intermittent PTH administration on bone volume, BMD, and bone cellular activity.

2.7.1 Continuous PTH Administration

The effects of PTH on bone metabolism is dependent upon the duration and frequency of exposure, with prolonged secretion or continuous PTH administration eliciting reductions in bone volume and microarchitecture [115, 125, 126] by constantly stimulating bone resorption. In this scenario, osteoclast-mediated resorption overtakes osteoblast-mediated bone formation [126, 127] and the differentiation of osteoprogenitor cells into osteoblasts is inhibited [128]. For example, continuous PTH 1-34 administration diminished alkaline phosphatase (i.e., a protein secreted by osteoblasts during bone formation) and augmented the bone resorption biomarker, C-terminal telopeptide [126, 129].

Imbalances in bone cellular activity initiate reductions in bone volume, BMD, and bone strength. For example, 12 days of continuous administration of PTH 1-34 reduced trabecular BV/TV by 45% in the tibia, which corresponded with a 40% reduction in Tb.N when compared to animals treated with PTH in an intermittent fashion [115]. The unfavorable effects of continuous PTH secretion are not limited to bone volume. For example, older patients (69-81 years old) with hyperparathyroidism (i.e., chronically elevate serum PTH) demonstrated significantly lower BMDs at multiple locations of the hip (i.e., total hip, femoral

neck, and trochanter) vs. the control group with normal levels of serum PTH [130]. In contrast, 2 weeks of continuous PTH 1-34 infusion did not alter BMD in the femur, tibia or L5 vertebra, but reduced trabecular connectivity by 40% in comparison to mice administered a vehicle [131]. Moreover, strength of the femoral neck declined following 8 hours of continuous PTH 1-34 infusion vs. strength in vehicle-treated rats [125]. Therefore, these data suggest that continuous PTH administration induces bone catabolism through the imbalance of bone cellular activity, which reduces bone volume, BMD, and bone strength.

2.7.2 Intermittent PTH Administration

Intermittent PTH administration is osteogenic, and a well-known anabolic agent used to treat conditions of low bone mass [118, 120]. Intermittent PTH administration has regulatory effects on bone cellular communications and bone remodeling [120] and augments bone volume by stimulating osteoblast-mediated bone formation, while having no effect on [124] or reducing [38] osteoclast-mediated bone resorption. Previous investigations have demonstrated the anabolic effects of intermittent PTH administration on bone in human subjects [119, 132] and in animal models [38, 39, 121-123]. For example, elderly, postmenopausal women are at an increased risk of developing bone diseases such as osteopenia and osteoporosis [133]. Often, intermittent PTH administration is prescribed to alleviate these declines in bone mass [134]. Eighteen months of intermittent PTH

administration augmented spine and hip BMD by ~7% and ~2%, respectively, in 2532 postmenopausal women vs. the placebo-treated group [119]. In addition, 24 months intermittent PTH 1-34 administration improved BMD and BV/TV at the iliac crest in an elderly population with osteoporosis vs. values in a similar population without PTH treatment [132].

Improved bone parameters following intermittent PTH administration in rodent models have been conducted utilizing various experimental models such as fracture healing [123], ovariectomy-induced bone loss [121, 122], and under normal physiological conditions [38, 39]. For example, 2 month-old rats treated intermittently with PTH 1-34 during fracture healing had augmented femoral BMD (32% and 54%, respectively), bone mineral content (46% and 74%, respectively) and strength (61% and 119%, respectively) following 28 and 42 days [123]. In addition, ovariectomy in mature (6-7 months-old) rats induced osteoporosis in the tibial metaphysis [121, 122]. Following 4 and 8 weeks of intermittent PTH 1-34 administration in these ovariectomized animals, trabecular BV/TV was restored by 66% and 69%, respectively, coinciding with improved bone formation rates vs. vehicle-treated, ovariectomized controls [121, 122].

The anabolic effects of PTH treatment have also been demonstrated in the young, healthy skeleton. Despite a shorter duration (i.e., 2 weeks) of intermittent PTH 1-84 administration in young (3-5 months) male Wistar rats, data demonstrated increases in trabecular BV/TV, Tb.Th, and Tb.N. and reduced Tb.Sp

[39]. Bone histomorphometric analysis of the femur revealed increased osteoblast activity (i.e., Ob.S/BS) and reduced osteoclast activity (i.e., Oc.S/BS) [38, 39]. A shorter time course (i.e., 7 days) of intermittent PTH 1-34 administration in middle-aged (16-month-old) Sprague-Dawley rats failed to alter BV/TV, which remained similar to vehicle-treated animals [135]. However, bone static properties related to bone formation including osteoid perimeter, osteoblast perimeter, and the number of osteoblasts were augmented by ~7-fold [135]. Therefore, when administered intermittently, PTH promotes bone anabolism by enhancing osteoblast activity and bone formation. Despite the documented changes in bone cellular activity, the underlying mechanisms are only partially understood [118, 126].

2.8 Effects of PTH Administration on the Bone Vascular Network

Intermittent PTH administration increases bone metabolism and with the rise in metabolism, enhanced bone blood flow is requisite. Thus, the influences of PTH on bone blood vessels have been recently investigated, since this may be another mechanism contributing to improved bone mass. These investigations assessed the vasodilator capacity of bone blood vessels, bone blood flow or perfusion, bone vascular density, and the spatial location of bone marrow blood vessels following either a bolus dose or intermittent administration [38, 39, 67, 136-138]. The next sections of this document discuss these parameters.

2.8.1 Increased Vasodilator Capacity of Bone Blood Vessels

The effects of PTH on systemic vasodilator function has been reported for decades [139, 140]. For example, cumulative doses of PTH 1-34 relaxed aortic strips up to 50% [141] and renal artery rings by 78% [142], reducing the vasoconstrictor effectiveness of norepinephrine and methoxamine. What has not been investigated until recent years has been the effects of intermittent PTH administration on bone vascular morphology and function. Similar to observations following bolus administration, intermittent PTH administration has systemic effects on vasodilator capacity. For example, 15 days of treatment augmented vasorelaxation of aortic rings to acetylcholine in old (22-24 months) Fischer-344 rats [143], highlighting beneficial aspects of this treatment on the cardiovascular system.

Parathyroid hormone receptor 1 (PTH1R), the primary receptor for PTH and parathyroid hormone-related peptide (PTHrP), is present on bone cells such as osteoblasts [144, 145]. Parathyroid hormone receptor 1 has also been demonstrated on human umbilical vein endothelial cells [146, 147], vascular endothelial cells [148], and vascular smooth muscle cells [149-151]. Comparable to the other vascular beds, bolus delivery of PTH and intermittent administration promote vasodilation of bone blood vessels. For example, in response to cumulative doses of PTH 1-84, PTH 1-34 and PTHrP 1-34 delivered *in vitro*, the femoral PNA from young (3-5 months) male Wistar rats demonstrated moderate-to-strong vasodilator

responsiveness [136]. The vasodilator response was the least and most robust to PTH-1-34 and PTHrP 1-34, respectively, and vasodilation to all 3 analogs of PTH were primarily mediated through vascular endothelial cell function [136].

Intermittent PTH administration over a period of time elicits alterations in endothelial cell function that become independent of PTH. For example, two weeks of intermittent PTH 1-84 administration not only augmented bone volume, but increased vasodilation by 33% in the femoral PNA of young (3-5 months-old) male Wistar rats vs. vehicle-treated controls [39]. Similar to vasodilator mechanism in response to bolus administration, vasodilation resulted primarily from NO-mediated, endothelium-dependent mechanisms in response to acetylcholine, suggesting that PTH treatment enhanced endothelial cell responsiveness to factors other than PTH (i.e., acetylcholine) [39]. Similar responses were demonstrated in older (22-24 months) rats, whereby 2 weeks of intermittent PTH 1-34 administration improved NO-mediated, endothelium-dependent vasodilation in response to acetylcholine [67]. Enhanced vasodilator function in response to intermittent PTH administration would presumably aid in the delivery of blood flow to bone, a requisite for enhanced bone metabolism. Thus, the benefits of this treatment include enhanced bone volume and improved endothelium-dependent vasodilator capacity; i.e., two aspects of physiological function impaired with advancing age.

2.8.2 Increased Bone Perfusion

Improvements in vasodilation induced by PTH should enhance the ability of the femoral PNA (and other bone blood vessels) to deliver blood and nutrients to sites of bone formation. Investigators have examined the effects of PTH on bone blood flow [138, 152, 153] and perfusion [137, 154] to address this theory. To date, there is no clear consensus. For example, a bolus dose of PTH 1-34 administered to healthy canines reduced bone blood flow by 50%, 15 minutes following injection [152]. Bone blood flow returned to baseline by 240 minutes post-injection [152]. In addition, a bolus dose of bovine PTH 1-34 reduced femoral and tibial blood flows in rats [153]. In contrast, increased ($p<0.05$) perfusion was observed in the femoral diaphysis [137, 154] and whole hindlimb [137] following bolus injections of PTH in mice. Whole hindlimb perfusion was considerably increased (>30%; $p<0.05$) within 10 to 15 minutes following the injection of PTH 1-34; however these measurements included the entire hindlimb and skeletal blood flow could not be distinguished from blood flow to other tissues [137]. In this same series of investigations, the authors measured perfusion of the femoral diaphysis with a procion red fluorescent tracer, revealing increased (32%; $p<0.05\%$) presence of the tracer in the cortical shell [137]. These findings suggest that the whole hindlimb and intracortical perfusion to the femoral diaphysis were improved by bolus administration of PTH.

Similar to bolus administration, the effects of intermittent PTH administration on bone blood flow and perfusion are variable. In post-menopausal women, skeletal perfusion was assessed by injection of radiopharmaceutical ^{99m}Tc-methylene diphosphonate [138], which approximates bone blood flow [155]. Following 3 and 18 months of intermittent PTH 1-34 administration, (i.e., a recombinant form of PTH 1-84), blood flow to the entire skeleton was augmented by 22% at 3 months and by 34% at 18 months. Additionally, 14 days of intermittent PTH 1-84 administration in 4 month-old female C57BL/6J mice increased tibial perfusion by 27% vs. vehicle-treated controls [154], whereas 4 weeks of intermittent PTH 1-34 administration did not augment tibial and hindlimb perfusion in 10-12 week-old BALB/c male mice [137]. Differences in these findings may relate to the age and sex of the animals and the use of different PTH analogs.

Overall, the effects of bolus and intermittent PTH administration on bone blood flow and perfusion are divergent. Future studies that standardized the duration of administration, the age and sex of the animals, and the PTH analogs are required before a clear understanding is obtained.

2.8.3 Angiogenesis

Tissue blood flow depends on the vasomotor properties of blood vessels as well as the vascular density (i.e., the number of blood vessels) supplying the tissue. In regards to the effects of intermittent PTH administration on the bone vascular

system, it may also serve to alter the number of blood vessels within bone [154, 156, 157]. To date, the effects of PTH on bone angiogenesis is not well-clarified. Intermittent PTH 1-34 administration stimulated angiogenesis in the cerebral circulation by promoting the release of endothelial progenitor cells [158]. Thus, it is reasonable that PTH would play a similar role in the bone vascular network. In fact, some studies demonstrated angiogenesis in the skeletal system after PTH administration. For example, continuous [154, 156] and intermittent [154] PTH administration augmented bone vascular density by 40% and 22%, respectively, in the L1-L3 vertebrae and in the tibial metaphysis of mice [154, 156]. Similarly, intermittent PTH 1-84 administration for 14 days in mice augmented ($p<0.05$) bone vascular density in the tibial metaphysis and diaphysis by 16% and 34%, respectively. [154]. In contrast, a lower number of bone marrow blood vessels in the tibial metaphysis was observed following 15 and 30 days of treatment in young (3-4 months) rats [38].

The lack of angiogenesis observed in this study did not translate into a lack of effect on the entire bone vascular system. Interestingly, the bone marrow blood vessels were spatially closer to sites of new bone formation (i.e., osteoid seams) following PTH treatment [38]. More poignantly, the proximity of the smallest bone marrow blood vessels ($\leq$ 29 μm in diameter) were closer to the sites of bone remodeling (i.e., 43 μm vs. 67 μm, respectively) in PTH-treated vs. control animals [38]. These findings suggest that spatial relocation of the smallest bone marrow

blood vessels (i.e., capillaries and small arterioles and venules) occurred following intermittent PTH administration. Such a spatial relocation may serve to facilitate nutrient exchange between blood vessels and bone [38]. In accord, bone remodeling observed in human bone biopsies revealed an augmented number of capillaries within 50 μm from BRCs; i.e., sites of high metabolic activity [159]. Thus, alterations in the bone vascular network following intermittent PTH administration may include the relocation of bone marrow blood vessels. Potential mechanisms by which this may occur are discussed in the next section.

2.9 Matrix Metalloproteinase-9

Matrix Metalloproteinase-9 (MMP-9) belongs to the MMP protein family, which is accountable for tissue remodeling [160]. For example, MMP-9-mediated extracellular matrix (ECM) degradation initiates vascular smooth muscle remodeling primarily by the breakdown of type IV collagen in the matrix of blood vessels [161]. In addition, several studies highlight the role of MMP-9 as a chemotactic agent that stimulates cellular migration [162-169]. Intriguingly, it has been demonstrated that MMP-9 is up-regulated in bone following intermittent PTH 1-34 administration in rats [170, 171]. This section illustrates the potential role(s) of MMP-9 in bone with intermittent PTH administration.

2.9.1 The Role of MMP-9

In the skeleton, ECM remodeling in bone results in mineralization and MMPs are associated with these processes [172]. Bone ECM is composed of mineralized hydroxyapatite (i.e., crystals of calcium-phosphate) and type I collagen as the organic ECM [172]. Type I collagen-specific MMPs (i.e., MMP-2 and MMP-14) are theorized to be of primary importance in the degradation of the ECM [173] and formation of the lacunar-canaliculi network [174] in bone. However, MMP-9 (a.k.a. Gelatinase B) plays a significant role in degrading collagen type IV, the basement membrane, and the ECM of blood vessels [161]. Although MMP-9 selectively degrades collagen type IV in the ECM of blood vessels, previous research by McClelland et al. demonstrated that MMP-9 was up-regulated during intermittent PTH administration and was associated with bone anabolism [171]. In addition, MMP-9 is expressed by many cells that are involved in bone remodeling; i.e., osteoclasts [175], osteoblast and osteocytes [171]. The augmented presence of MMP-9 and bone anabolism suggest a potential role for MMP-9 in remodeling of the bone ECM with intermittent PTH administration.

Besides its role in matrix degradation of blood vessels, MMP-9 stimulates the migration and homing of many different cell types, which include those involved in bone remodeling [162, 163, 169], megakaryocytes [165], endothelial progenitor cells [164], hematopoietic progenitor cells [166], and vascular endothelial [176] and smooth muscle cells [167]. In many cases, MMP-9 is induced

by stromal cell-derived factor-1 in order to recruit the target cell. Stromal cell-derived factor-1 released from bone cells and endothelial cells serves to increase mRNA expression of MMP-9, promoting migration of megakaryocytes and pre-osteoclasts towards bone [165, 169]. When MMP-9 is inhibited or deleted, impediment of trans-endothelial migration of megakaryocytes occurs [165], as well as diminished osteoclast recruitment in cultured bone tissue [162] and in the tibiae of embryos [163].

Matrix Metalloproteinase-9 also promotes the migration vascular smooth muscle cells [167, 176]. For example, overexpression of MMP-9 in a transgenic mouse model promoted vascular smooth muscle cell migration to levels similar to wild-type mice [167]. Furthermore, in a cell culture study, abolishment of MMP-9 attenuated vascular smooth muscle cell migration in comparison to wild-type cells, while reactivation of MMP-9 restored migration of vascular smooth muscle cells to levels observed in wild-type vascular smooth muscle cells [177]. Vascular endothelial cells are also influenced by MMP-9 [176]. For example, hypoxia-induced up-regulation of MMP-9 in fibrosarcoma cells enhanced endothelial cell migration, while inhibition of MMP-9 prevented endothelial cell migration [176]. Similarly, inhibition of MMP-9 in human microvascular endothelial cells restrained their migration, as assessed with cell migration assays [178]. These data suggest that MMP-9 not only plays a pivotal role in ECM degradation and remodeling but also influences migration and homing of various cell types. Since endothelial cell

migration is a required step for angiogenesis [178], these data support the notion that the role of MMP-9 in vascular ECM degradation may be associated with angiogenesis and neovascularization.

2.9.2 PTH and MMP-9

Previous studies demonstrated an increased expression of MMP-9 in chondrocytes [170] and long bones [171] following intermittent PTH administration. In the skeleton, PTH induced MMP-9 expression from osteoblasts, osteocytes, and bone marrow cells [171]. As previously mentioned, overexpression of MMP-9 following PTH administration aided in osteoclast recruitment by augmenting the migration of pre-osteoclasts [162, 163, 169]. Thus, bone remodeling induced by intermittent PTH administration may relate to increased expression of MMP-9 as the following manner: 1) partial bone ECM remodeling by MMP-9 [161], 2) differentiation of pre-osteoclasts into osteoclasts [162, 163, 169], and 3) the initiation of bone remodeling. Given that bone marrow blood vessels were observed closer to bone forming sites in PTH-treated vs. control rats [38], MMP-9 may be involved in this spatial relocation. Although more investigations are needed in the context, we theorize that augmented secretion of MMP-9 from bone cells during intermittent PTH administration may serve to attract bone marrow blood vessels towards the surface of bone.

2.10 Conclusions

Advancing age is associated with bone loss and bone vascular dysfunction in the form of diminished vasodilator capacity, vascular rarefaction, and bone marrow blood vessel ossification. Parathyroid hormone is the only FDA approved bone anabolic treatment for osteoporosis and, coincidently, it improves endothelium-dependent vasodilation of bone blood vessels. To date, the influence of intermittent PTH administration on skeletal blood flow and angiogenesis remain controversial; however, relocation of bone marrow blood vessels closer to sites of bone formation has been documented. Given enhanced release MMP-9 during intermittent PTH administration, this chemotactic agent may participate in the relocation of bone marrow blood vessels. In total, the consensus remains that intermittent PTH administration has beneficial effects on bone and bone vascular function. However, given its role in bone formation, intermittent PTH administration may serve to exacerbate bone marrow blood vessel ossification. Currently, no studies have examined this potential negative consequence of PTH treatment. Given the increased volume of ossified bone marrow blood vessels with advancing age and the treatment of elderly individuals with PTH, this question is vital to address. Therefore, the aims of this dissertation are to assess bone marrow blood vessel ossification, bone marrow blood vessel relocation, bone vascular density, MMP-9 density, area and localization, bone microarchitecture, and bone static and dynamic properties in relation to age (Mature vs. Middle-Aged) and

short-term (5- and 10-days) intermittent PTH 1-34 administration. Five and ten days of intermittent PTH 1-34 administration in mice is equivalent to approximately six and twelve months of treatment, respectively, in the clinical populations. Findings of this dissertation will confirm the time point when bone loss and bone vascular impairment commence across the life span. Also, the short duration of intermittent PTH 1-34 administration will validate that alterations in the bone vascular system precede the changes in bone following PTH treatment.

2.11 Specific aims

Hypothesis 1: Bone marrow blood vessel ossification will be higher in Middle-Aged vs. Mature mice and short-term intermittent PTH 1-34 administration will augment bone marrow blood vessel ossification in both groups.

Specific Aim 1. Determine the volume and thickness of ossified bone marrow blood vessels in Mature (6-8 month-old) and Middle-Aged (10-12 month-old) C57BL/6 mice following short-term (5 and 10 days) intermittent PTH 1-34 administration.

Hypothesis 2.1: Vascular density will be reduced in Middle-Aged vs. Mature mice and 5- and 10-days of intermittent PTH 1-34 administration will have no effect on vascular density in either group.

Hypothesis 2.2: Distances between bone marrow blood vessels and bone will be increased in Middle-Aged mice, and intermittent PTH 1-34 administration will decrease the distances similar to those observed in Mature mice.

Specific Aim 2. Determine bone marrow blood vessel density and the distances between bone marrow blood vessels and trabecular bone in Mature (6-8 month-old) and Middle-Aged (10-12 month-old) C57BL/6 mice following 5- and 10-days of intermittent PTH 1-34 administration.

Hypothesis 3.1: PTH will up-regulate the release of MMP-9 near the trabecular bone surface in both Mature (6-8 month-old) and Middle-Aged (10-12 month-old) mice.

Specific Aim 3. Determine MMP-9 density, area and localization in relation to trabecular bone and bone marrow blood vessels in Mature (6-8 month-old) and Middle-Aged (10-12 month-old) mice following 5- and 10-days of intermittent PTH 1-34 administration.

Due to the short duration of intermittent PTH administration, I do not anticipate any changes in bone microarchitecture and bone static and dynamic properties. However, in addition to assessing the alterations in the bone vascular system, the following bone-related parameters have also been assessed:

1) Trabecular bone microarchitecture in the distal femoral metaphysis: bone volume-to-total volume ratio (BV/TV, %), trabecular number (Tb.N,

/mm^2), trabecular thickness (Tb.Th, μm), and trabecular separation (Tb.Sp, μm).

2) Cortical bone parameters at the femoral mid-shaft: cortical volume fraction (Ct.BV/TV, %), and cortical thickness (Ct.Th, μm).
3) Trabecular bone static properties related to osteoblast activity: osteoid surface to bone surface ratio (OS/BS, %) and osteoblast surface to bone surface ratio (Ob.S/BS, %).
4) Trabecular bone static properties related to osteoclast activity: osteoclast surface to bone surface ratio (Oc.S/BS, %).
5) Trabecular bone dynamic properties related to bone formation: single-labeled surface per bone surface ratio (sLS/BS, %), double-labeled surface per bone surface ratio (dLS/BS, %), mineralizing surfaces to bone surface ratio (MS/BS, %), mineral apposition rate (MAR), and bone formation rate to bone surface ratio (BFR/BS, μm^3/μm^2/day).

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Chapter 3

Short-Term Intermittent PTH 1-34 Administration and Bone Marrow Blood Vessel Ossification in Mature and Middle-Aged C57BL/6 Mice

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3.1 Abstract

INTRODUCTION: Intermittent parathyroid hormone (PTH) administration augments bone and progressive bone marrow blood vessel (BMBV) ossification occurs with advancing age. Since intermittent PTH administration augments bone, it may also serve to increase BMBV ossification. I assessed the influence of 5- and 10-days of intermittent PTH 1-34 administration on trabecular and cortical bone and BMBV ossification in mature (6-8 mon; n=30) and middle-aged (10-12 mon; n=30) male and female C57BL/6 mice.

MATERIALS AND METHODS: Mice were divided accordingly: control (CON) and 5-days (5dPTH) and 10-days (10dPTH) of PTH. Mice were given PBS (50 µl) or PTH 1-34 (43 µg/kg/d) for 5- and 10-consecutive days. Trabecular bone microarchitecture (i.e., BV/TV [%], Tb.Th [µm], Tb.N [/mm^2], and Tb.Sp [µm]) was assessed in the distal femoral metaphysis and cortical bone parameters (i.e., Ct.BV/TV [%] and Ct.Th [µm]) at the femoral mid-shaft. BMBV ossification (i.e., ossified vessel volume [OsVV, %] and ossified vessel thickness [OsV.Th, µm]) was assessed in the medullary cavity of the femoral shaft. All parameters were determined by µCT. At this sample size, no gender-related differences were observed so female and male data were pooled.

RESULTS: There were no main effects nor interactions for trabecular and cortical bone parameters. OsVV tended (p=0.057) to be higher (0.18±0.04% vs. 0.09±0.02%, respectively) and OsV.Th was higher (p<0.05; 17.4±1.6 µm vs.

12.1±1.4 μm, respectively) in Middle-Aged vs. Mature mice. OsVV was not altered, but ossified vessels tended (p=0.08) to be thicker in 10dPTH (17.6±2.0 μm) vs. CON (12.5±1.7 μm). No interactions were observed for OsVV and OsV.Th.

CONCLUSIONS: This is the first report of ossified BMBV in C57BL/6 mice. The increased OsV.Th in Middle-Aged mice coincides with previous reports of increased OsVV in aged rats. The tendency of augmented OsV.Th in 10dPTH suggests that this treatment may ultimately impair the patency of bone marrow blood vessels.

3.2 Introduction

The vascular system is crucial for the optimal functioning of bone and bone marrow. Blood vessels deliver O_2, nutrients and systemic hormones to these tissues and remove waste products [1-3]. Additionally, immune cells [4] and precursor cells involved in bone remodeling are produced in the marrow and blood vessels are responsible for the transport of these cells [5, 6]. Thus, blood vessels are fundamental for bone remodeling [7-9] and, in regards to hematopoiesis, play a role in stem cell niches [10, 11].

Given the various roles of the bone vascular system in the day-to-day functioning of a healthy skeleton, it has been theorized that dysfunction in the bone vascular network is highly associated with dysfunction in bone and bone marrow [12-17]. For example, diminished bone blood flow or perfusion [12, 14-16, 18] and

impaired vasodilator function of bone blood vessels [14, 16, 19] are associated with reduced skeletal mass in rats and humans [14-16, 18]. Further, age-related rarefaction of blood vessels coincides with bone loss and augmented bone marrow adiposity [20, 21]. In a rat aging model, we recently described a novel pathology whereby bone marrow blood vessels (BMBV) progressively and theoretically convert into bone (i.e., BMBV ossification) with advancing age [21]. Ossified blood vessels were also present in amputated long bones from elderly patients with arteriosclerotic vascular disease and peripheral vascular disease with cellulitis [21]. These findings confirm the translation of this disease to the human condition.

Ossification of BMBV is characterized by osteocyte lacunae on the abluminal surface and may result from a transition of vascular endothelial and/or smooth muscle cells into osteogenic phenotypes [21]. Additionally, the etiologies of this disease may be attributable to the bone marrow microenvironment and the release of pro-inflammatory cytokines from the resident cells [22]. Regardless of the etiologies, ossified BMBV lose vasomotor function (i.e., vasodilator and vasoconstrictor activity) and have reduced or abolished patency, resulting in "microvascular dead space" within bone [21]. "Microvascular dead space" presumably contributes to the age-related declines in skeletal blood flow or perfusion [12, 14-16, 18] and reversal in direction of skeletal blood flow [23], potentially impacting delivery of nutrients, systemic hormones and precursor cells to bone and bone marrow [13, 16, 21]. Such a progressive pathology may ultimately

contribute to the diminished bone mass observed with advanced age [12, 14, 16, 21].

Intermittent parathyroid hormone (PTH) administration is osteogenic and a well-known anabolic agent used to treat conditions of low bone mass [24, 25]. Intermittent PTH administration has regulatory effects on bone cellular communications and remodeling [25, 26]. For example, intermittent PTH administration augmented osteoblast differentiation and activated bone lining cells [27], in addition to reducing osteoblast apoptosis [28]. While the effects of intermittent PTH administration on bone have been well documented, PTH also elicits vasodilation of blood vessels in various organs [29-31], including the skeleton [17, 32]. This physiological effect may be particularly important for the bone vascular network in terms of enhanced blood flow delivery during the augmented bone metabolism induced by intermittent PTH administration. In fact, augmented bone volume following 15 days of intermittent PTH 1-84 administration in young rats was accompanied by enhanced endothelium-dependent vasodilation of the femoral principal nutrient artery (PNA) [17]; i.e., the primary conduit for blood flow to long bones [1]. Additionally, intermittent PTH administration was demonstrated to augment bone vascular density (i.e., blood vessel number) and skeletal perfusion [33, 34]. In contrast, bone vascular density was lower in PTH-treated vs. control rats following 15- and 30-days of intermittent PTH 1-84 administration [35].

The totality of data suggest a beneficial physiological outcome for several bone vascular parameters (i.e., vasodilator capacity of blood vessels, blood flow and angiogenesis) following intermittent PTH administration that would facilitate bone accrual. However, given that intermittent PTH administration is effective at eliciting bone formation, it may also and unfortunately promote or exacerbate BMBV ossification. The long-term consequences of such a result would serve to further reduce the ability of bone blood vessels to deliver flow to the aging skeleton. Since BMBV ossification has been demonstrated in an aging rat model and in human amputated long bones [35], I hypothesized that BMBV ossification would be observed in Mature and Middle-Aged C57BL/6 mice and that it would be more severe in the Middle-Aged animals. Additionally, previous investigations revealed that physiological stimuli influence the bone vascular system prior to impacting bone [33, 36]. Thus, I chose short duration protocols (i.e., 5 and 10 days) to capture these sequences of events. Therefore, I hypothesize that short-term intermittent PTH 1-34 administration would not be of sufficient duration to alter trabecular and cortical bone parameters but would enhance BMBV ossification in Mature and Middle-aged C57BL/6 mice.

3.3 Materials and Methods

All animal experiments were conducted and performed according to the protocols approved by University of Delaware and University of Texas at Arlington

Institutional Animal Care and Use Committees and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Mature (6-8 month-old) and Middle-Aged (10-12 month-old) female and male C57BL/6 mice were obtained from a mouse colony at the University of Delaware. They were housed in standard cages in a temperature- (23±2 °C) and light-controlled (12h/12h light/dark) room. Tap water and regular mouse chow were provided *ad libitum*.

3.3.1 Intermittent Parathyroid Hormone Administration and Sample Preparation

According to body mass, Mature and Middle-Aged mice were divided into the following six groups: 1) Mature control (Mature CON; n=10); 2) Mature with five days of intermittent PTH 1-34 administration (Mature 5dPTH; n=10); 3) Mature with ten days of intermittent PTH 1-34 administration (Mature 10dPTH; n=10); 4) Middle-Aged CON (Middle-Aged CON; n=10); 5) Middle-Aged 5dPTH (n=10); and 6) Middle-Aged 10dPTH (n=10). According to treatment, mice received subcutaneous injections of either 43 μg/kg/day of PTH 1-34 (ProSpec, East Brunswick, NJ) for five and ten consecutive days or 50 μl/day of phosphate buffered saline as a vehicle for ten consecutive days. A dose of 43 μg/kg/day of PTH 1-34 is molecularly equivalent to 100 μg/kg/day of PTH 1-84 [37]. PTH and the vehicle were administrated at the same time daily. After completion of the protocol, all mice were anesthetized by inhalation of isoflurane (2.5% to O_2

balance). Mice were sacrificed via myocardial removal and left femora were collected. The femora were cleaned and fixed overnight in 4% paraformaldehyde at 4ºC. Femora were subsequently stored in 70% ethanol at -20ºC until scanning by micro-computed tomography (MicroCT).

3.3.2 Micro-Computed Tomography (MicroCT) Scans and Analyses

MicroCT scans were performed using a high-resolution µCT 35 (Scanco Medical, Brüttisellen, Switzerland). Femora were scanned at a resolution of 10 µm at 55kVp. Trabecular bone microarchitecture was determined from 60 slices in the distal femoral metaphysis, beginning 600 µm inferior from an anatomically defined region of the growth plate. The following trabecular bone microarchitectural parameters were calculated: bone volume/total volume ratio (BV/TV, %), trabecular thickness (Tb.Th, µm), trabecular number (Tb.N, /mm^2) and trabecular separation (Tb.Sp, µm). Cortical bone parameters were determined from 50 slices at the femoral mid-shaft and the following parameters were calculated: cortical bone volume (Ct.BV/TV, %) and cortical thickness (Ct.Th, µm). In addition, the femoral shaft was analyzed to assess ossified BMBV in the marrow space. Care was taken so that trabecular and cortical bone was not included in the analysis. Thus, the analysis began where trabecular bone disappeared at the end of the secondary spongiosa in the proximal metaphysis and continued just prior to the beginning of the secondary spongiosa at the distal metaphysis. The following

parameters were calculated: ossified vessel volume (OsVV, %) and ossified vessel thickness (OsV.Th, μm).

3.3.3 Statistical Analysis

Data were analyzed by two-way ANOVA with SPSS statistical software (version 24; IBM, Armonk, NY) to determine significant main effects (i.e. age and treatment) and interactions for the following: trabecular bone microarchitecture, and cortical bone and ossified BMBV parameters. One-way ANOVA was used to examine differences in body mass. Student-Newman-Keuls (SNK) post-hoc tests were performed to assess group differences. The significance level was set at $p \leq 0.05$. Tendencies for significant differences ($p \leq 0.10$) are reported. Data were expressed as a mean ± standard error (M ± SE).

3.4 Results

3.4.1 Mouse Characteristics

Body mass did not differ among groups (Mature CON, 31±2g; Mature 5dPTH, 32±2g; Mature 10dPTH, 30±1g; Middle-Aged CON, 30±1g; Middle-Aged 5dPTH, 31±1g; and Middle-Aged 10dPTH, 31±1g). Since no gender-related differences were observed at this sample size, female and male data were pooled and analyzed according to group.

3.4.2 Effects of Age and Intermittent PTH Administration on Trabecular and Cortical Bone Parameters

There were no main effects for age or treatment nor any significant interactions observed for the trabecular and cortical bone parameters. As anticipated, trabecular bone microarchitecture (i.e., BV/TV, Tb.N, Tb.Th, Tb.Sp) and cortical bone parameters (Ct.BV/TV and Ct.Th) did not differ with PTH treatment (Table 3.1). Figure 3.1 illustrates representative 3D reconstructions of trabecular bone microarchitecture in the secondary spongiosa of the distal femoral metaphysis and the cortical shell at the femoral mid-shaft.

Table 3.1. Trabecular and cortical bone parameters following short-term intermittent PTH 1-34 administration in Mature and Middle-Aged mice.

Trabecular Bone

	Mature CON	**Mature 5dPTH**	**Mature 10dPTH**	**Middle-Aged CON**	**Middle-Aged 5dPTH**	**Middle-Aged 10dPTH**
BV/TV (%)	2.2±1.1	2.5±1.0	2.2±0.8	2.3±0.9	3.7±1.2	4.0±1.0
Tb.Th (μm)	27.2±3.3	26.1±2.3	26.4±1.8	28.2±2.0	26.8±3.6	29.4±2.1
Tb.N /mm^2	0.7±0.3	0.9±0.3	0.7±0.2	0.7±0.2	1.1±0.3	1.2±0.3
Tb.Sp (μm)	22227.4± 12679.5	4225.5± 1850.6	4923.0± 2593.1	3987.4± 1482.4	22649.9± 16864.9	5810.4± 4636.8

Cortical Bone

	Mature CON	**Mature 5dPTH**	**Mature 10dPTH**	**Middle-Aged CON**	**Middle-Aged 5dPTH**	**Middle-Aged 10dPTH**
Ct.BV/TV (%)	30.0±0.8	29.2±1.2	32.0±0.8	28.8±0.7	26.5±3.1	29.6±1.1
Ct.Th (μm)	172.0±5.4	174.7±7.5	181.8±5.4	174.3±6.0	163.1±18.9	179.6±3.9

Values are mean ± SE.

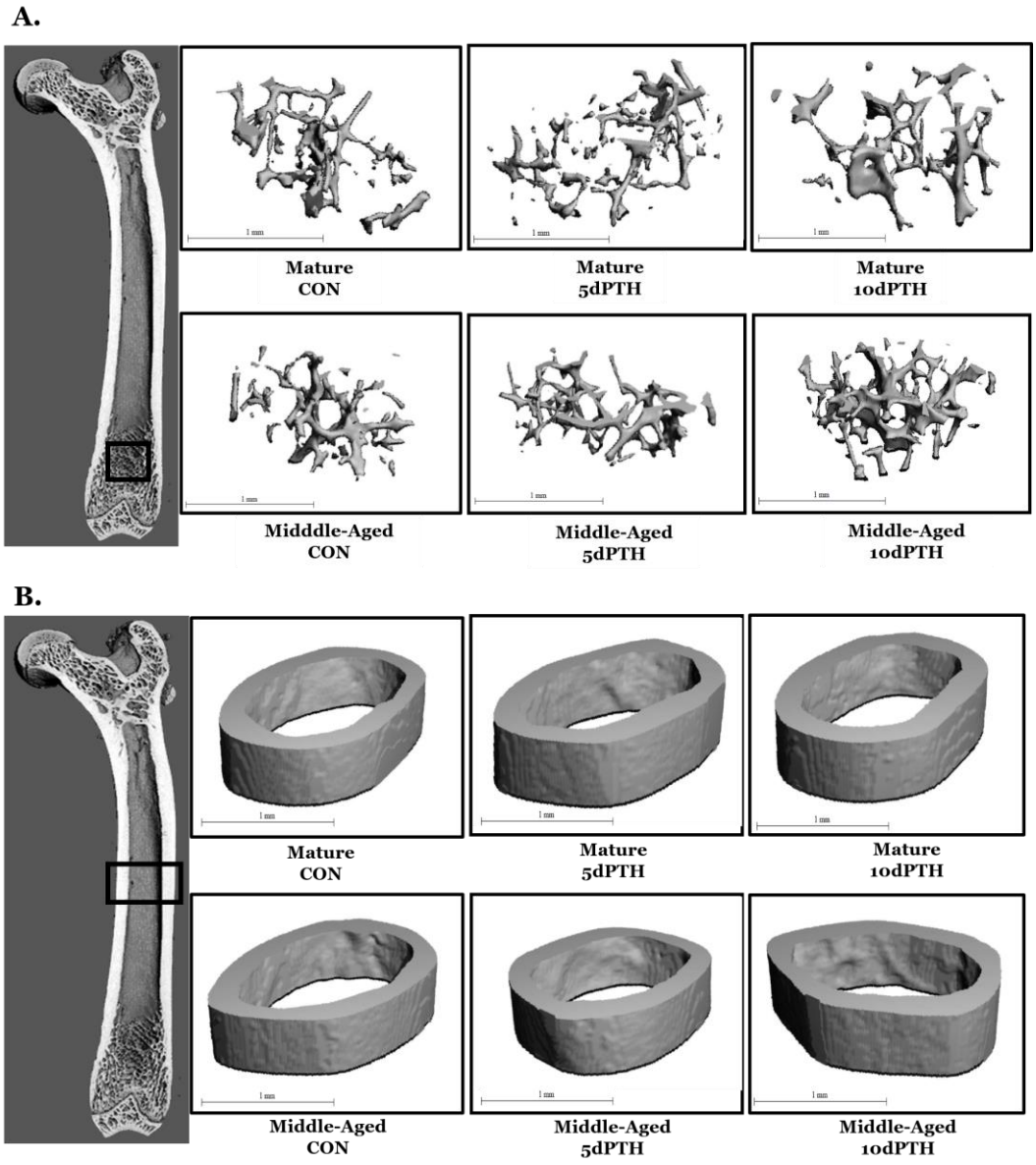

Figure 3.1. Representative images of (A) trabecular bone microarchitecture and (B) cortical thickness in Mature and Middle-Aged CON, 5dPTH, and 10dPTH mice.

3.4.3 Effects of Age and Intermittent PTH Administration on Bone Marrow Blood Vessel Ossification

As hypothesized, ossified BMBV were present in both Mature and Middle-Aged C57BL/6 mice. Figure 3.2 depicts representative images of ossified bone marrow blood vessels for each group. No significant interactions were observed for the ossified vessel parameters (i.e., OsVV and OsV.Th); however, some main effects for age were found. OsVV tended ($p=0.057$) to increase with advancing age (Figure 3.3A). Further, ossified vessels were 44% thicker ($p<0.05$) in Middle-Aged vs. Mature mice (Figure 3.3B). Additionally, there were no significant differences in OsVV according to treatment (Figure 3.3C). However, ossified vessels tended ($p=0.08$) to be 41% thicker following 10 days of intermittent PTH administration (Figure 3.3D).

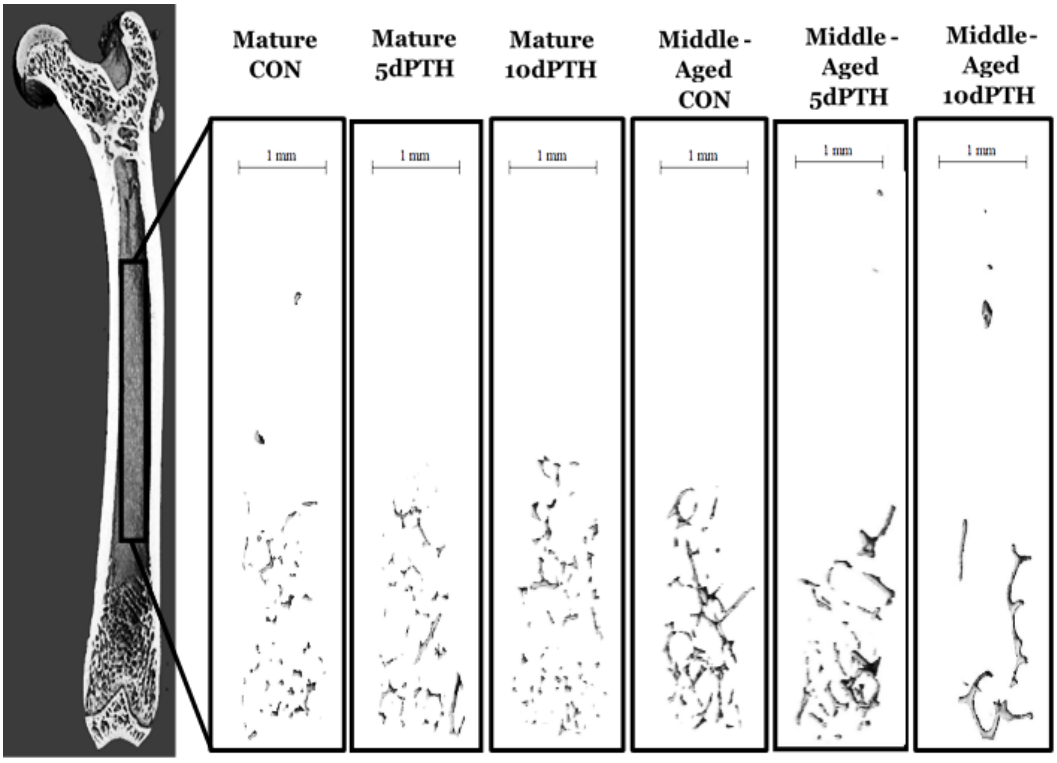

Figure 3.2. Representative images of bone marrow blood vessel (BMBV) ossification in mature and middle-aged CON, 5dPTH, and 10dPTH mice. These images demonstrate the presence of the BMBV ossification in both Mature and Middle-Aged female and male C57BL/6 mice, regardless of the treatment.

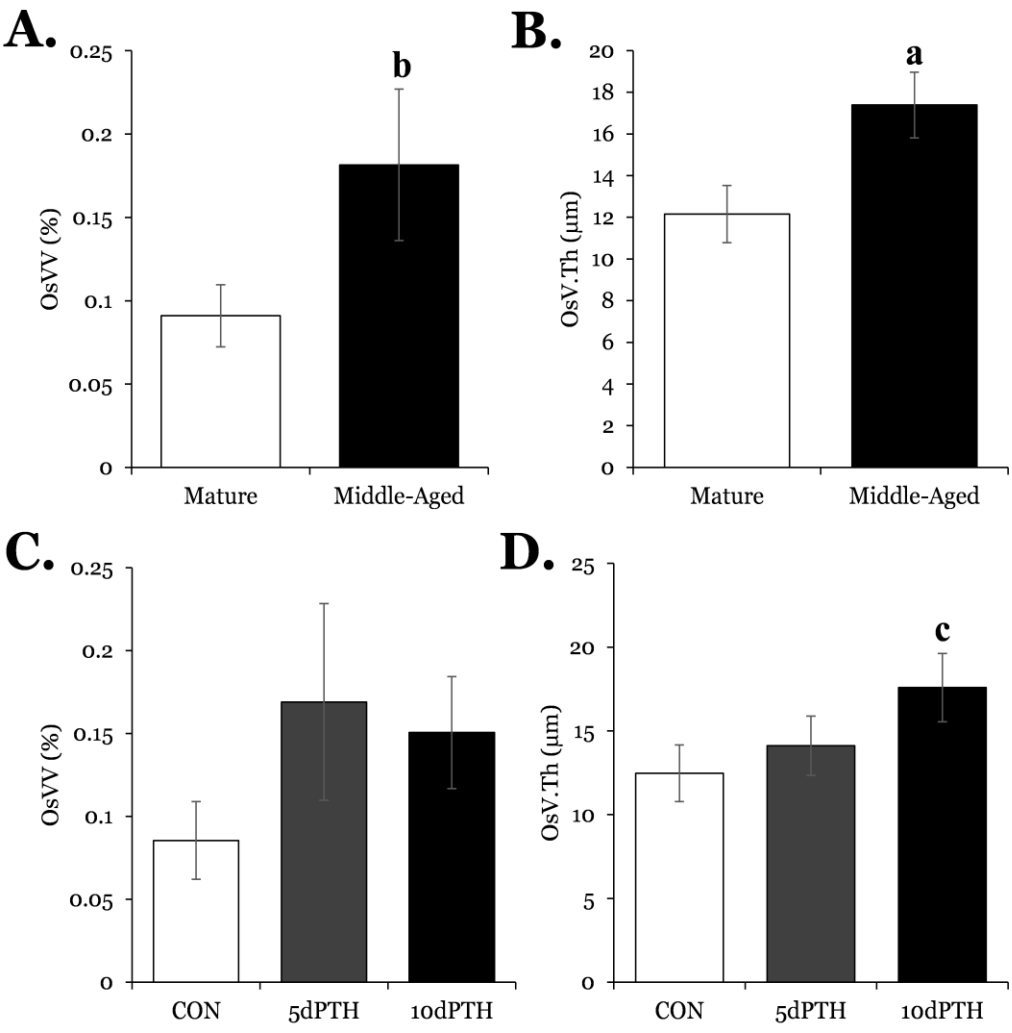

Figure 3.3. Main effects of age (panels A & B) and intermittent PTH administration (panels C & D) on ossified bone marrow blood vessel (BMBV) parameters (i.e., OsVV, and OsV.Th). (A) OsVV tended (p=0.057) to be higher in Middle-Aged vs. Mature mice. (B) OsV.Th was higher (p>0.05) in Middle-Aged mice vs. Mature mice. (C) OsVV was not altered with short-term (5- and 10-days) PTH 1-34 administration. (D) OsV.Th tended (p=0.08) to be higher in 10dPTH

vs. CON. Values are means ± S.E. [a]denotes significant difference vs. Mature ($p<0.05$), [b]denotes a tendency for difference vs. Mature (p=0.057), [c]denotes a tendency for difference vs. CON (p=0.08).

3.5 Discussion

This investigation verified that ossified BMBV are present in Mature and Middle-aged female and male C57BL/6 mice (Figure 3.2), confirming that various species (i.e., mice, rats, and humans) develop this vascular pathology (current data and [21]). In addition, ossified BMBV were thicker in Middle-Aged vs. Mature animals (Figure 3.3), indicating continued bone formation and accrual on these blood vessels as a function of advancing age. To our knowledge, these are the first data to report the presence of ossified BMBV in C57BL/6 mice.

These data confirm our previous reports of progressive ossification as a function of advancing age in rats [21]. Ossification of the bone vasculature, characterized by osteocyte lacunae and osteoid seams, presumably serves to enhance "microvascular dead space" [21]. "Microvascular dead space" indicates that ossified BMBV have reduced patency, are incapable of normal vasomotor activities (i.e., vasodilation and/or vasoconstriction), and thus are deficient in the regulation of bone blood flow [21]. Further, ossified BMBV were observed in amputated long bones from elderly individuals [21], highlighting the prevalence of this disease in humans. Even though not statistically significant (p=0.057), OsVV

doubled in a matter of months (i.e., from maturity to middle-age) and ossified vessels became thicker ($p<0.05$) during this time frame. Both of these findings support previous claims of progressive ossification related to the aging process [21]. Of note are the alterations in vascular parameters that proceeded the changes in bone.

Trabecular and cortical bone parameters did not differ between the Mature and Middle-Aged groups; however, ossified vessels were thicker in the older mice. Skeletal maturity in mice occurs between 4-6 months of age [38, 39]. Following skeletal maturity, age-related declines in bone mass occur, but the rate of decline is species and strain specific [40]. The lack of a 4-6 month age group in the current investigation precludes determination as to whether trabecular and cortical bone was already reduced by 6-8 months of age. However, during the period between 6-12 months, the lack of change in trabecular and cortical bone parameters coincided with increased mineralization on the bone vascular network.

Coupled with BMBV ossification, other vascular pathologies occur with advancing age that may have a tremendous impact on bone and bone marrow. For example, declines in trabecular bone volume and endothelium-dependent vasodilation of the femoral PNA were observed at 22-24 months vs. 4-6 months in male Fischer-344 rats [14, 16, 19]. In addition, the loss of bone vascularity has been reported [21, 41], which may coincide with diminishment of a capillary subtype theorized to regulate angiogenesis [42]. For example, bone blood vessel rarefaction

occurred between 4 and 7 months of age in 129sv/CD1 mice, coinciding with reduced trabecular bone volume [40]. However, no changes in vascular density were observed in the C57BL/6 mice [40], highlighting potential strain-specific alterations. Bone vascular rarefaction with advancing age has also been reported in humans, whereby the number of arterial capillaries (per 100 mm^2 of tissue) declined from 10-20 to 30-50 years and the number of sinusoids (per 100 mm^2 of tissue) declined from 10-20 to 30-50 and again at >70 years [20]. Since PTH influences the cardiovascular system, several authors have speculated beneficial alterations in the bone vascular network following its administration [17, 22, 33, 35, 36]. Thus, such therapies may serve to reverse the aforementioned age-related declines in the bone vascular network.

Both acute and intermittent PTH administration influences bone blood vessels. For example, vasodilation to cumulative doses of various PTH analogs (i.e., PTH 1-84, PTH 1-34 and PTHrP 1-34) was observed in the femoral PNA [17, 32], with vasodilation being most robust to PTHrP 1-34 [32]. Further, single applications of PTH transiently augmented skeletal blood flow and perfusion [36, 43]. When administered intermittently over several weeks or months, PTH augmented skeletal perfusion in mice and humans [33, 44]. Further, PTH 1-84 relocated the smallest BMBV closer to bone forming sites [35]. This spatial relocation presumably aided in directing blood flow to areas of bone undergoing remodeling and maximized nutrient exchange [35]. Additionally, 14 days of

intermittent PTH 1-84 administration augmented bone vascular density profiles in hind limb long bones of mice [33]; however, bone vascular density was lower in PTH-treated rats following 15 and 30 days of treatment [35]. Lastly, intermittent administration of PTH 1-84 and PTH 1-34 improved endothelium-dependent vasodilation in PNAs from young and old rats, respectively [17, 22] and enhanced the marrow microenvironment in old (22-24 mon) rats [22]. Overall, these studies highlight beneficial modifications of the bone vascular network with acute application or intermittent PTH administration for 14-30 days.

While the beneficial effects of PTH have been well documented in terms of enhancing the vasomotor properties of bone blood vessels and increasing bone vascular density and skeletal perfusion under certain circumstances, the potential negative effects presented herein deserve further attention. The questions to be addressed in future experiments are whether the beneficial effects of intermittent PTH administration on bone outweigh the potential long-term, negative consequences on bone blood vessels. Intermittent PTH administration is bone anabolic [17, 35, 45-47]; however, clinically-speaking, the treatment is of a limited duration (i.e., $\leq$ 2 years) [48]. Thus, once treatment is arrested, what long-term consequences remain for the bone vascular system? In this investigation, intermittent PTH 1-34 administration tended to increase OsV.Th (Figure 3) in as short as 10 days; i.e., at a time point when trabecular and cortical bone were unaltered. This finding may prove particularly concerning for individuals who are

prescribed and for physicians prescribing PTH treatment for low bone mass, as it is suggestive of an unfavorable consequence with longer durations of treatment.

The long-term consequences of intermittent PTH administration on the volume of ossified vasculature may depend upon the initial volume of the ossified vasculature at the onset of treatment. Unfortunately, the initiation of intermittent PTH administration in human subjects often occurs in states of advanced age, potentially coinciding with an already augmented "microvascular dead space" [21]. Coupled with BMBV ossification, age-related declines in skeletal blood flow [12, 14, 16, 49, 50], bone vascular rarefaction [20, 21], and reduced vasodilator capacity of the femoral PNA [14, 16, 19, 51] have already been documented. The improvements reported in bone blood vessel density [33], skeletal perfusion [33, 34] and vasodilator function [17, 22] with intermittent PTH administration may serve to initially offset any negative consequences of enhanced "microvascular dead space" in bone. However, it is possible that augmented ossification of BMBV with PTH treatment would eventually exacerbate vasomotor decline, reduce bone blood vessel patency, diminish skeletal blood flow, and ultimately obstruct bone accrual once intermittent PTH administration has been medically arrested.

Unsurprisingly, intermittent PTH 1-34 administration did not alter trabecular and cortical bone parameters in Mature and Middle-Aged mice. This finding no doubt reflects the short administration period (i.e., 5 and 10 days). This timeframe was chosen to examine the prompt effects of PTH administration on the

bone vascular network. Unfortunately, the osteogenic effects of PTH on ossified BMBV occurred more rapidly than the osteogenic effects on bone, suggesting a greater sensitivity of bone blood vessels to PTH.

In contrast, longer durations of intermittent PTH administration coincides with trabecular and cortical bone accrual [33, 52, 53]. For example, intermittent PTH administration for 14 to 28 days augmented cortical and trabecular bone in the tibiae and femora of C57BL/6 mice [33, 52, 53]. On the contrary, 4 weeks of intermittent PTH treatment in 10-12-week-old BALB/c mice increased cortical bone volume but had no effect on trabecular bone volume in the distal femur and L5 vertebra [36]. Thus, the anabolic actions of intermittent PTH are wide-ranging and have been suspected to vary according to the strain of the animal [36, 54].

Similarly, the varying effects of intermittent PTH administration are observed in human patients as well. Overall, intermittent PTH administration is an effective treatment for osteoporosis; however, skeletal responses to PTH can be divergent from individual to individual [55]. A longitudinal study using high-resolution peripheral quantitative computed tomography (HR-pQCT) documented divergent effects of PTH 1-34 and PTH 1-84 in postmenopausal osteoporotic patients [56]. In this investigation, PTH 1-34 augmented cortical porosity so that cortical density was reduced in the radius and tibia following treatment, whereas PTH 1-84 was associated with weakened bone strength [56]. In addition, one cohort study using QCT demonstrated that 1 year of intermittent PTH 1-84 administration

augmented spinal volumetric BMD 60% above baseline in 20% of the study participants, while one-fifth of participants experienced no change or reduced BMD [57]. The reasons for such a large and divergent response to PTH treatment were not fully understood.

The divergent skeletal responses may be partially attributable to the volume of ossification in the patients at the initiation of treatment. In other words, the lack of bone anabolism or decline in bone parameters observed in some individuals with intermittent PTH administration may be etiologically vascular in origin. We have demonstrated progressive BMBV ossification as a function of advancing age in rats [21] and currently demonstrated the potential for enhanced BMBV ossification in mice with PTH treatment. Under these circumstances, the expansion of "microvascular dead space" in bone would serve to further impair vascular function and limit nutrient and factor delivery via blood flow if declines in vessel patency outstrip bone angiogenesis [33] and the improved vasodilator capacity of "normal" bone blood vessels [17, 22].

In conclusion, this is the first study to report the presence of ossified vessels in C57BL/6 mice. Further, current and previous [21] results demonstrate that BMBV ossification is a pathology observed in rodent and human long bones. Additionally, 10 days of intermittent PTH 1-34 administration tended to increase the thickness of ossified BMBV, potentially exacerbating the pathology. Even though PTH is a treatment for osteoporosis, it may eventually impact the patency

of BMBV by augmenting ossification and increasing the "microvascular dead space" in bone. While effective at bone anabolism, the long-term (i.e., following the arrest of treatment) consequences of intermittent PTH administration on the patency of BMBV may eventually outweigh the benefits on bone.

3.6 Conflicts of Interest

Seungyong Lee and Rhonda D. Prisby declare that they have no conflicts of interest.

3.7 Acknowledgements

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Chapter 4

Short-Term Intermittent PTH 1-34 Administration Stimulates Angiogenesis and Matrix Metalloproteinase-9 Secretion in Femora of Mature and Middle-Aged C57BL/6 Mice

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4.1 Abstract

INTRODUCTION: Intermittent parathyroid hormone (PTH) administration increases bone volume and matrix metalloproteinase (MMP)-9 secretion. In addition, intermittent PTH alters bone blood vessels by increasing vasodilator capacity, augmenting bone perfusion, and relocating bone marrow blood vessels closer to osteoid seams. Discrepancies exist, however, as to whether intermittent PTH elicits angiogenesis. Since MMP-9 participates in cellular homing and migrating, I theorized that it aides in relocating bone marrow blood vessels. This study examined the influence of short-term (i.e., 5- and 10-days) intermittent PTH on angiogenesis, MMP-9 secretion, and the distance between blood vessels and bone in mature (6-8mon; n=30) and middle-aged (10-12mon; n=30) male and female C57BL/6 mice.

METHODS: Mice were divided accordingly: control (CON), 5-days intermittent PTH (5dPTH) and 10-days intermittent PTH (10dPTH). Mice were given a placebo (i.e., PBS; 50 µl/d) or PTH 1-34 (43 µg/kg/d). Right femora were collected and prepared for histology. Frontal sections (5µm) were triple-immunolabeled to identify endothelial cells (i.e., anti-CD31), vascular smooth muscle cells (i.e., anti-αSMA), and MMP-9 (i.e., anti-MMP9). Sections of the distal femoral metaphysis were imaged to determine vascular density, MMP-9 density, area and localization, and blood vessel distance to bone. Blood vessels were analyzed according to diameter: 1-29µm, 30-100µm and 101-200µm. Trabecular bone microarchitecture (i.e., BV/TV [%], Tb.Th [µm], Tb.N [/mm^2], and Tb.Sp [µm]) and bone static (i.e., OS/BS, %, Ob.S/BS, % and Oc.S/BS, %) and dynamic (MAR, µm/day; sLS/BS, %; dLS/BS, %; MS/BS, % and BFR, $\mu m^3/\mu m^2$/day) properties were examined.

RESULTS: Gender-related differences were not observed with this sample size, so female and male data were pooled. The density of 1-29μm and 30-100μm CD31-labeled blood vessels was significantly (p<0.05) higher and tended (p=0.055) to be higher, respectively, in 10dPTH vs. 5dPTH and CON. Further, 30-100μm and 101-200μm αSMA-labeled blood vessels tended (p<0.10) to be higher in 10dPTH vs. 5dPTH. MMP-9 was augmented (p<0.05) in 10dPTH vs. the other groups and MMP-9 was closer (p<0.05) to 1-29μm blood vessels and furthest (p<0.05) from trabecular bone. No differences were observed in distances between blood vessels and bone, bone microarchitecture, and bone static and dynamic properties.
CONCLUSION: In conclusion, bone angiogenesis occurs by 10 days of iPTH (i.e., quicker than changes in trabecular bone), coinciding with augmented MMP-9 secretion closer to the smallest (1-29μm) blood vessels.

4.2 Introduction

Intermittent parathyroid hormone (PTH) administration is a well-known osteoanabolic agent used to treat osteoporosis [1, 2] since it plays an essential regulatory role in bone remodeling [1]. In addition, evidence in the literature demonstrates positive effects of intermittent PTH administration on the bone vascular system [3-7]. For example, bolus doses of PTH rapidly enhanced bone blood flow and bone perfusion [7, 8]. *In vitro* vasodilation of the femoral principal nutrient artery (PNA; the major conduit for blood flow to long bones [9]) was augmented in young Wistar rats with cumulative doses of PTH 1-34, PTH 1-84,

and parathyroid hormone-related peptide (PTHrP) 1-34 [4]. Chronic administration of PTH also elicits vascular alterations. Two weeks of intermittent PTH 1-84 administration increased the vasodilator capacity of femoral PNAs in young (3-5 months) Wistar rats, which coincided with augmented bone volume [5]. Similarly, age-related declines in vasodilator capacity and bone volume in old (22-24 months) Fischer-344 rats were partially restored to values similar to young (4-6 months) rats following 2 weeks of intermittent PTH 1-34 administration [10].

In addition to bone perfusion and vasodilator capacity, angiogenesis can also be impacted by intermittent PTH administration. For example, vascular density in the tibial metaphysis was augmented following 14 days of intermittent PTH 1-84 administration in mice, coinciding with increased trabecular thickness and bone formation rate, but not bone volume [6]. In contrast, bone vascular density was lower in young PTH-treated (15- and 30-days) vs. control Wistar rats [3]. Discrepancies in these data may relate to the different species examined (i.e., mouse vs. rat). Since intermittent PTH administration is often prescribed to elderly individuals, it may also serve to enhance bone angiogenesis and vascular density in this population. For example, bone marrow capillaries and sinusoids were reduced as a function of advancing age in humans [11] and, following fracture healing, blood vessel numbers in the tibial mid-shaft were reduced in 18-month vs.1-month-old mice [12]. Thus, the influence of intermittent PTH administration on bone angiogenesis as a function of advancing age remains to be ascertained.

Alterations in bone angiogenesis following intermittent PTH administration may relate to the upregulation of matrix metalloproteinase (MMP)-9 [13, 14], which is proangiogenic [15]. For example, 5 days of intermittent PTH administration increased the expression of MMP-9 by osteoblasts and osteocytes in the primary spongiosa of the tibial metaphysis of young rats [15]. Further, since extracellular matrix (ECM) remodeling is crucial for vascular outgrowth, MMP-9-mediated ECM remodeling is closely associated with angiogenesis and neovascularization [14]. In addition, MMP-9 regulates endothelial progenitor cell (EPC) number, activity, and migration to regions of angiogenesis [13].

While demonstrated to increase MMP-9 secretion by bone cells [15], intermittent PTH administration failed to augment blood vessel number in young male Wistar rats [3], leading one to speculate that MMP-9 may have additional functions. For example, MMP-9 plays a role in the migration of various cell types (i.e., osteoclasts [16, 17], endothelial cells [17], and vascular smooth muscle cells [18]). Due to its role in cellular migration and homing [19, 20], and its upregulation following intermittent PTH administration, it may also participate in the spatial relocation of bone marrow blood vessels. For example, 15- and 30-days of intermittent PTH 1-84 administration relocated bone marrow blood vessels closer to bone forming sites [3]. Unfortunately, MMP-9 secretion was not analyzed in this study [3]; however, it is plausible that MMP-9 contributed to this response. Given the duality of its known functions (i.e., angiogenesis and cellular migration and

homing), further clarification on the role of MMP-9 with intermittent PTH administration is warranted. Therefore, I sought to determine the effects of short-term (5- and 10-days) intermittent PTH 1-34 administration on bone marrow blood vessel density, MMP-9 density, area and localization, the distance between bone marrow blood vessels and trabecular bone surfaces, trabecular bone microarchitecture, and bone static and dynamic properties in Mature and Middle-Aged mice. Since previous investigations reported rapid bone vascular responses in comparison to bone [6, 7, 21], short-term (5- and 10-days) intermittent PTH 1-34 administration should allow for examination of bone vascular alterations absent the alterations in bone. Based upon previous data demonstrating lower blood vessel number in bone with advanced age [11, 12] and following intermittent PTH administration [3], I hypothesize that Middle-Aged mice will have a lower number of bone marrow blood vessels vs. Mature mice and intermittent PTH 1-34 administration will have no effect on bone vascular density. Since PTH stimulates MMP-9 secretion [15], I hypothesize that MMP-9 in the distal femoral metaphysis will be higher with PTH treatment. Finally, given the role of MMP-9 in cellular migration and homing [16-19], it may play a similar role in the spatial re-localization of bone marrow blood vessels closer to bone. Thus, I anticipate that MMP-9 will be localized closer to trabecular bone vs. the bone marrow blood vessels following PTH treatment.

4.3 Materials and Methods

The experiment was carried out in accordance with the protocols approved by University of Delaware and the University of Texas at Arlington Institutional Animal Care and Use Committees and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Mature (6-8 month-old) and Middle-Aged (10-12 month-old) female and male C57BL/6 mice were housed in standard cages in a temperature- (23±2 °C) and light-controlled (12h/12h light/dark) room. Tap water and regular mouse chow were provided ad libitum.

4.3.1 Intermittent Parathyroid Hormone Administration and Sample Preparation

According to body mass, 60 mature and middle-aged mice were divided into the following six groups: 1) Mature control (Mature CON; n=10), 2) Mature with five days of intermittent PTH 1-34 administration (Mature 5dPTH; n=10), 3) Mature with ten days of intermittent PTH 1-34 administration (Mature 10dPTH; n=10), 4) Middle-Aged CON (Middle-Aged CON; n=10), 5) Middle-Aged with five days of intermittent PTH 1-34 administration (Middle-Aged 5dPTH; n=10), and 6) Middle-Aged with ten days of intermittent PTH 1-34 administration (Middle-Aged 10dPTH; n=10). Each group had an equal distribution of female and male mice. For the PTH treated groups, mice received either 43 µg/kg/day of PTH

1-34 or a vehicle (i.e., phosphate buffered saline; 50 µl/day) subcutaneously for five and ten consecutive days. A dose of 43 µg/kg/day of PTH 1-34 is molecularly equivalent to 100 µg/kg/day of PTH 1-84 [22]. Injection of either PTH or the vehicle was performed at the same time daily. Following the completion of the protocol, all mice were anesthetized by inhalation of isoflurane (2.5% to O_2 balance) and were sacrificed via cardiectomy. Right femora were collected, cleaned of soft tissue, fixed overnight in 4% paraformaldehyde at 4°C, and decalcified with a Cal-Ex™ (Fisher Scientific, Hampton, NH, United State). The decalcification solution was changed every 2-3 days and a decalcification end-point indicator was used to ensure that femora were appropriately decalcified. Femora were subsequently processed for paraffin embedding and frontal sections of the distal femoral metaphysis were cut with a microtome (Leica RM2255, Leica Microsystems, Buffalo Grove, Illinois, United State).

4.3.2 Immunolabeling

Endothelial cells, vascular smooth muscle cells, and MMP-9 were identified by triple-immunolabeling. Briefly, bone sections were deparaffinized and antigen retrieval was accomplished with 10 mM Citrate Buffer (pH 6.0) and a heat-induced antigen retriever (2100 Retriever, Aptum Biologics Ltd., Southampton, United Kingdom). Subsequently, the sections were incubated with the IgG blocking solution (M.O.M mouse IgG blocking reagent, Vector Laboratories, Burlingame,

CA, United States) and protein blocking solution (M.O.M Diluent Protein blocker, Vector Laboratories, Burlingame, CA, United State) to block non-specific protein binding sites. Sections were then immunolabeled with primary antibodies and fluorescently labeled with secondary antibodies. To detect endothelial cells, the following primary and secondary antibodies were utilized: a rat anti-CD31 (Abcam #ab56299, Abcam, Cambridge, United Kingdom) and a goat, anti-rat IgG-Alexa Fluor 488 conjugated (Abcam #ab150157, Abcam, Cambridge, United Kingdom). To detect vascular smooth muscle cells, the following primary and secondary antibodies were utilized: rabbit anti-alpha smooth muscle actin (αSMA; Abcam #ab124964, Abcam, Cambridge, United Kingdom) and a goat, anti-rabbit IgG-Alexa Fluor 647 conjugated (Abcam #ab150079, Abcam, Cambridge, United Kingdom). To identify MMP-9, the following primary and secondary antibodies were utilized: a mouse anti-MMP-9 purified antibody (Abcam #ab119906, Abcam, Cambridge, United Kingdom) and a goat, anti-mouse IgG-Alexa Fluor 594 conjugated (Abcam #ab150116, Abcam, Cambridge, United Kingdom). For each section, a negative control slide stained with only secondary antibodies were generated. To minimize the signal generated by autofluorescence of the bone marrow, sections were incubated in Sudan Black. Finally, slides were mounted with Prolong Gold with DAPI mounting solution (Cell Signaling Technology, Danvers, MA, United States) to detect nucleated cells.

Sections were analyzed with a fluorescent microscope (Olympus BX 51, Olympus Corporation, Tokyo, Japan). The negative control slides were used to eliminate background fluorescence. Subsequently, triple-immunolabeled sections were analyzed to detect CD31, αSMA, and MMP-9 without interference of a background signal. CD31, αSMA, and MMP-9 were distinguished with FIT-C, CY5, TRIT-C filters, respectively. In addition, bright-field images of trabecular bone were collected. Images were obtained with NIS Element software (NIS Elements, Nikon, Tokyo, Japan) and subsequently analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA).

4.3.3 Bone Vascular Density

The density of bone marrow blood vessels was determined by automated counting of CD31- and αSMA-immunolabeled bone marrow blood vessels. The blood vessels were quantified according to diameter (i.e., 1-29μm, 30-100μm, and 101-200μm) and normalized by tissue area (i.e., the number of blood vessels per mm^2). These vessel sizes were chosen to roughly estimate the type of blood vessels. For the sake of simplicity, the smallest blood vessels (1-29μm) are considered as capillaries, with the recognition that arterioles and venules may have diameters within this range. Blood vessels 30-100μm and 101-200μm in diameter were considered arterioles and venules and small arteries and veins, respectively, with

recognition that some designations may overlap according to individual vessel diameter.

4.3.4 Spatial Distribution of Bone Marrow Blood Vessels from Trabecular Bone

Two immunolabeled sections per mouse were used to determine the distances between CD31- and αSMA-labeled bone marrow blood vessels and trabecular bone surfaces. At least ten consecutive images (20X) of the secondary spongiosa in the femoral metaphysis were obtained per section. By use of Image J software, images of CD31-, αSMA-labeled bone marrow blood vessels and trabecular bone were converted into binary images. Vessels were analyzed according to diameter; i.e., 1-29μm, 30-100μm, and 101-200μm. Euclidian distance maps were generated for each analysis of interest (e.g., 1-29μm CD31-labeled bone marrow blood vessels and trabecular bone, etc.), and the distances between these two parameters were calculated automatically. Blood vessels >200μm in distance from trabecular bone surfaces were excluded from the analysis, as these vessels were considered too far away to provide blood flow to this section of bone. The same methodologies were employed to assess the distances between αSMA-labeled bone marrow blood vessels and trabecular bone surfaces.

4.3.5 MMP-9 Density, Area and Localization

Two immunolabeled sections per bone sample were used to assess MMP-9 density, area and localization. Similar to the blood vessel density measurements, MMP-9 density and area were determined by automated counting and assessment of area of the MMP-9-immunolabeled signal. These measurements were assessed in the secondary spongiosa of the distal femoral metaphysis. The MMP-9 density and area were normalized by tissue area (i.e., the density or area of MMP-9 per mm^2). To assess MMP-9 localization from blood vessels (i.e., 1-29μm, 30-100μm, and 101-200μm) and bone, distances between MMP-9 and CD31- and αSMA-labeled blood vessels and MMP-9 and trabecular bone were analyzed. At least ten consecutive images (20X) were acquired per section. Images were converted into binary images with Image J software. Bone marrow blood vessels were analyzed according to diameter and Euclidian distance maps were generated for the analyses of distances from MMP-9 to each diameter range of blood vessels and to trabecular bone. The distances were calculated automatically with the software. To assess whether MMP-9 was localized closer to trabecular bone vs. the blood vessels, age and treatment groups were pooled and distances were analyzed according to vessel diameter (i.e., 1-29μm, 30-100μm, and 101-200μm). MMP-9 localizations >200μm in distance away from blood vessels or bone was excluded from the analysis.

4.3.6 Bone Microarchitecture

Two paraffin sections were stained with Masson's Trichrome to assess bone microarchitecture (i.e., bone volume to total volume ratio (BV/TV, %), trabecular thickness (Tb.Th, µm), trabecular number (Tb.N, /mm^2), and trabecular separation (Tb.Sp, µm). The analyses were conducted in the secondary spongiosa of the distal femoral metaphysis with the OsteoMeasure bone histomorphometry analysis system (OsteoMetrics, Decatur, GA, United States).

4.3.7 Bone Static Properties

Masson's Trichrome sections were used to determine bone static properties related to osteoblast activity; i.e., osteoid surface to bone surface ratio (OS/BS, %) and osteoblast surface to bone surface ratio (Ob.S/BS, %). To assess bone static properties related to osteoclast activity and bone dynamic properties, another set of mature and middle-aged mice were divided (according to body mass) into the following groups: 1) Mature CON; n=10, 2) Mature 5dPTH; n=10, 3) Mature 10dPTH; n=10, 4) Middle-Aged CON; n=10, 5) Middle-Aged 5dPTH; n=10, and 6) Middle-Aged 10dPTH; n=10. Each group had an equal distribution of female and male mice. At sacrifice, femora were dissected, dehydrated and embedded in methylmethacrylate (MMA) as previously described [3]. Two 5-µm-thick sections per mouse were used for the histo-enzymatic reaction of Tartrate-Resistant Acidic Phosphatase (TRAP) and osteoclast surface to bone surface ratio (Oc.S/BS, %) was

measured. The analyses were conducted in the secondary spongiosa of the distal femoral metaphysis with the OsteoMeasure bone histomorphometry analysis system.

4.3.8 Bone Dynamic Properties

To analyze bone dynamic properties, mice received intraperitoneal injections of tetracycline (25 mg/kg body weight) 7 and 2 days prior to sacrifice. Bone dynamic properties were measured under UV light microscopy from two 5-μm-thick MMA embedded unstained bone sections per mouse. The following parameters were analyzed: mineral apposition rate (MAR, μm/day), double-labeled surface per bone surface ratio (dLS/BS, %), and single-labeled surface per bone surface ratio (sLS/BS, %). Mineralizing surfaces per bone surface ratio (MS/BS, %) was calculated with the following formula: dLS/BS + ½ sLS/BS. Bone formation rate (BFR/BS $\mu m^3/\mu m^2$/day) was derived from the product of MS/BS and MAR. The analyses were conducted in the secondary spongiosa of the distal femoral metaphysis with the OsteoMeasure bone histomorphometry analysis system.

4.3.9 Statistical Analysis

Vascular density, MMP-9 density, MMP-9 area, bone microarchitecture, and bone static and dynamic properties were analyzed with 2×3 (age x treatment)

ANOVAs. Distances between CD31- and αSMA-labeled blood vessels and trabecular bone, and MMP-9 and trabecular bone were analyzed with 2×3 ANOVAs. One-way ANOVAs were used to determine differences in body mass and MMP-9 localization in relation to CD31- and αSMA-labeled blood vessels and trabecular bone. Student-Newman-Keuls (SNK) post-hoc tests were performed to assess differences among groups. The significance level of $p<0.05$ was set *a priori*. Data were expressed as mean ± standard error (M ± SE). SPSS statistical software (version 25.0; IBM, Armonk, NY) was used for all analyses.

4.4 Results

4.4.1 Mouse Characteristics

Body mass did not differ among groups: Mature CON, 30±3g, Mature 5dPTH, 31±2g, Mature 10dPTH, 31±2g, Middle-Aged CON, 31±2g, Middle-Aged 5dPTH, 31±1g, and Middle-Aged 10dPTH, 30±2g. Since there were no gender-related differences observed with these sample sizes, female and male data were pooled and analyzed according to the group.

4.4.2 Effects of Age and Intermittent PTH Administration on Bone Vascular Density

Table 4.1 presents age-related data for vascular density. Age-related changes were not observed for neither the density of CD31- or αSMA-labeled blood

vessels. However, the density of 30-100µm CD31-labeled blood vessels tended ($p=0.064$) to be higher in Middle-Aged vs. Mature mice. In contrast to age, main effects for treatment were observed. Figure 4.1 depicts the densities of CD31- and αSMA-labeled blood vessels at 1-29µm, 30-100µm, and 101-200µm. The number of 1-29µm CD31-labeled blood vessels was higher ($p<0.05$) in 10dPTH vs. the other groups (Figure 4.1A). In accord, the density of 30-100µm CD31-labeled blood vessels tended to be higher ($p=0.055$) following 10 days of PTH treatment (Figure 4.1B), but the density of 101-200µm CD31-labeled blood vessels did not differ (Figure 4.1C). On the contrary, 1-29µm αSMA-labeled blood vessels did not differ among treatment (Figure 4.1D). However, the density of 30-100µm and 101-200µm αSMA-labeled blood vessels tended to be higher ($p=0.089$ and $p=0.083$, respectively) in 10dPTH vs. 5dPTH (Figure 4.1E and 4.1F). No significant interactions were observed for bone vascular density. Figure 4.1G depicts representative images of CD31- and αSMA-labeled blood vessels in the secondary spongiosa of the distal femur.

Table 4.1. The main effect of age on the density of CD31- and αSMA-labeled bone marrow blood vessels.

CD31	**Mature**	**Middle-Aged**
1-29µm	7.27 ± 0.45 /mm^2	7.65 ± 0.51 /mm^2
30-100µm	0.29 ± 0.03 /mm^2	0.40 ± 0.05 /mm^2 **a**
101-200µm	0.03 ± 0.01 /mm^2	0.05 ± 0.01 /mm^2
α-SMA	**Mature**	**Middle-Aged**
1-29µm	6.88 ± 0.38 /mm^2	6.86 ± 0.36 /mm^2
30-100µm	1.08 ± 0.05 /mm^2	1.01 ± 0.04 /mm^2
101-200µm	0.47 ± 0.03 /mm^2	0.44 ± 0.03 /mm^2

Values represent Means ± S.E. [a]p=0.064 vs. Mature.
1-29µm, 30-100µm, and 101-200µm represents vessel diameter.

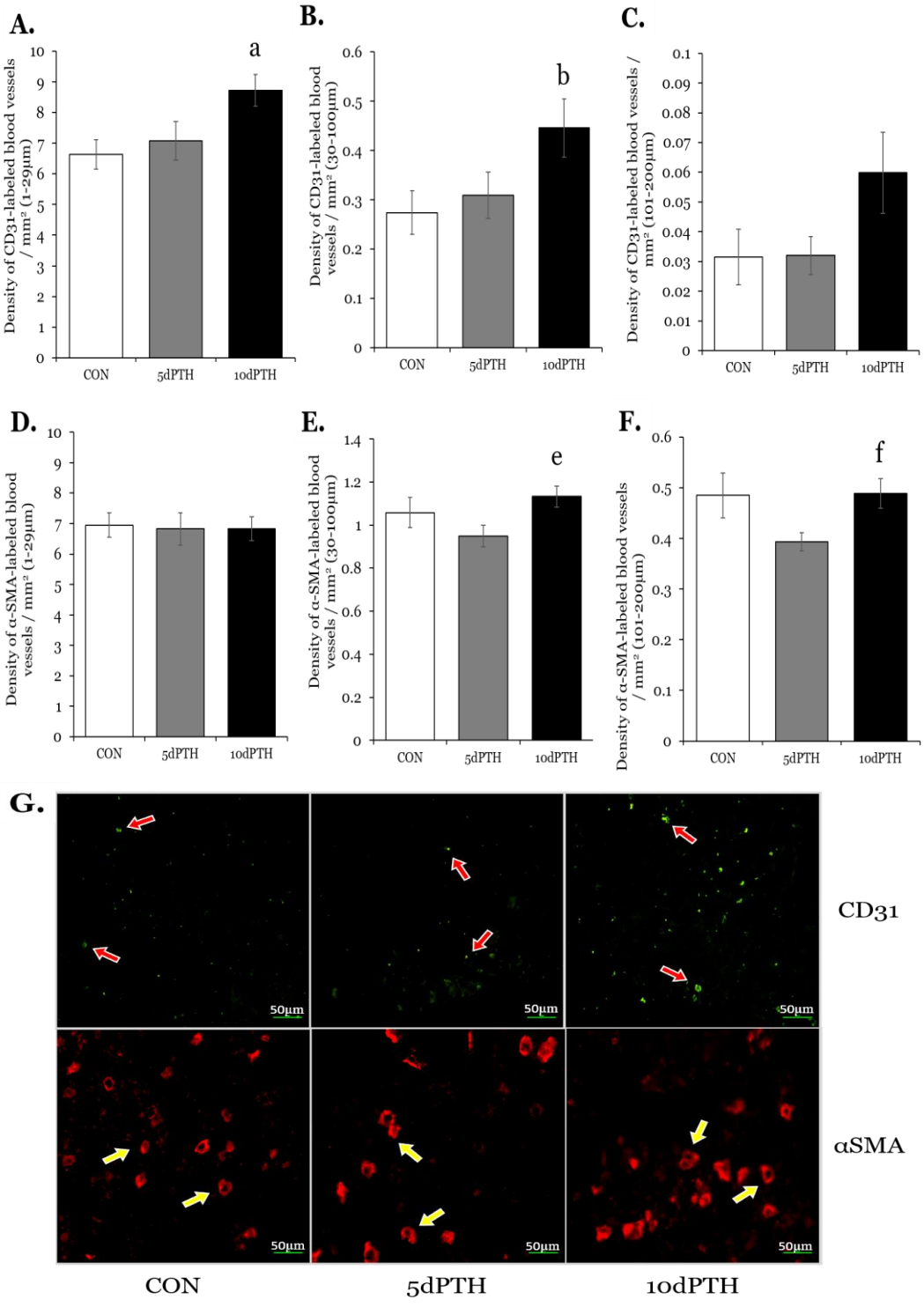
A.
B.
C.
D.
E.
F.
G.
a
b
e
f
Density of CD31-labeled blood vessels / mm² (1-29μm)
Density of CD31-labeled blood vessels / mm² (30-100μm)
Density of CD31-labeled blood vessels / mm² (101-200μm)
Density of α-SMA-labeled blood vessels / mm² (1-29μm)
Density of α-SMA-labeled blood vessels / mm² (30-100μm)
Density of α-SMA-labeled blood vessels / mm² (101-200μm)
CON
5dPTH
10dPTH
CD31
αSMA
50μm

Figure 4.1. The density of CD31- and αSMA-labeled bone marrow blood vessels following 5- and 10-days of intermittent PTH 1-34 administration. Density of CD31-labeled bone marrow blood vessels A) 1-29μm, B) 30-100 μm, and C) 101-200 μm in diameter. Density of αSMA-labeled bone marrow blood vessels D) 1-29μm, E) 30-100 μm, and F) 101-200 μm in diameter. Representative images for G) CD31-labeled bone marrow blood vessels (red arrows) and H) αSMA-labeled bone marrow blood vessels (yellow arrows) following 5- and 10-days of intermittent PTH treatment. Values are means ± S.E. [a]$p<0.05$ vs. CON and 5dPTH; [b]p=0.055 vs. CON and 5dPTH; [e]p=0.089 vs. 5dPTH; [f]p=0.083 vs. 5dPTH.

4.4.3 Effects of Age and Intermittent PTH Administration on MMP-9 Density and Area

There were no main effects for age (Figure 4.2A and 4.2B) nor any significant interactions observed for the density and area of MMP-9. Interestingly, there was a main effect for treatment, such that the density of MMP-9 was augmented ($p<0.05$) in 10dPTH vs. the other groups (Figure 4.2C). However, the area of MMP-9 (Figure 4.2D) did not differ. Figure 4.2E depicts representative images of MMP-9 in the secondary spongiosa of the distal femoral metaphysis.

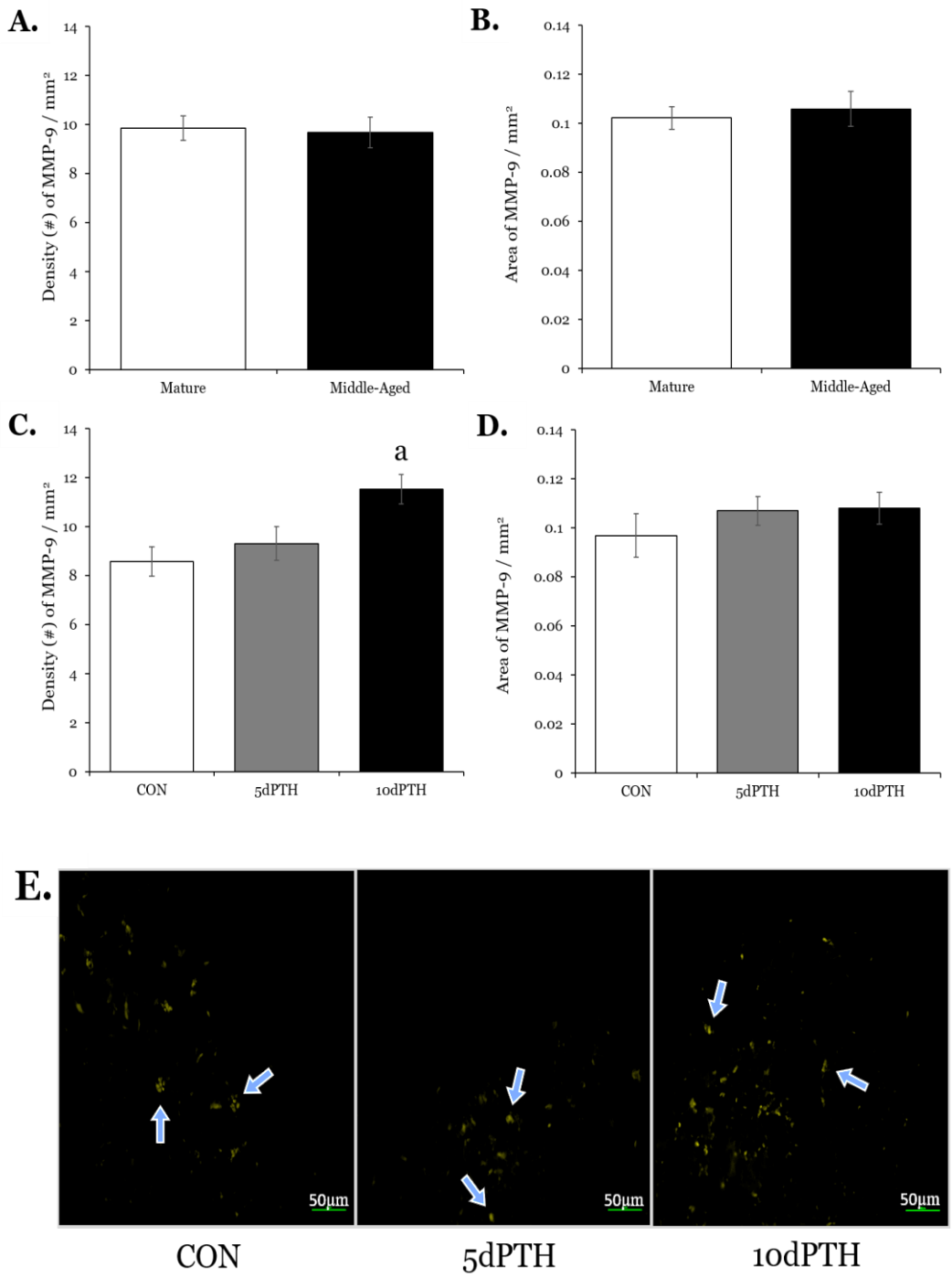
A.
Density (#) of MMP-9 / mm²
14
12
10
8
6
4
2
0
Mature
Middle-Aged
B.
Area of MMP-9 / mm²
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Mature
Middle-Aged
C.
Density (#) of MMP-9 / mm²
a
CON
5dPTH
10dPTH
D.
Area of MMP-9 / mm²
CON
5dPTH
10dPTH
E.
50μm
50μm
50μm
CON
5dPTH
10dPTH

Figure 4.2. Main effects for age and treatment on the density and area of MMP-9. No main effects for age were found in MMP-9 A) density and B) area. C) The density of MMP-9 was higher in 10dPTH vs. 5dPTH and CON. D) The area of MMP-9 did not differ among treatment. E) Representative images of MMP-9 (blue arrows) following 5- and 10-days of intermittent PTH administration. Values are means ± S.E. [a]$p<0.05$ vs. CON and 5dPTH.

4.4.4 Distances between Bone Marrow Blood Vessels and Trabecular Bone Surfaces

Distances of CD31- and αSMA-labeled bone marrow blood vessels from trabecular bone surfaces are presented in Table 4.2 and Figure 4.3. There were no significant main effects nor interactions. However, 101-200μm αSMA-labeled bone marrow blood vessels tended ($p=0.076$) to be closer to bone in 10dPTH vs. CON (Figure 4.3B).

Table 4.2. The main effect of age on the distance of CD31- and αSMA-labeled bone marrow blood vessels from trabecular bone.

CD31	**Mature**	**Middle-Aged**
1-29μm from Bone	128.5 ± 2.4 μm	125.1 ± 2.7 μm
30-100μm from Bone	152.8 ± 4.6 μm	146.8 ± 6.8 μm
101-200μm from Bone	130.1 ± 7.0 μm	135.3 ± 13.2 μm
α-SMA	**Mature**	**Middle-Aged**
1-29μm from Bone	129.2 ± 2.7 μm	124.1 ± 2.3 μm
30-100μm from Bone	130.6 ± 2.2 μm	130.4 ± 3.4 μm
101-200μm from Bone	126.7 ± 5.6 μm	126.2 ± 5.0 μm

Values represent Means ± S.E.
1-29μm, 30-100μm, and 101-200μm represents vessel diameter.

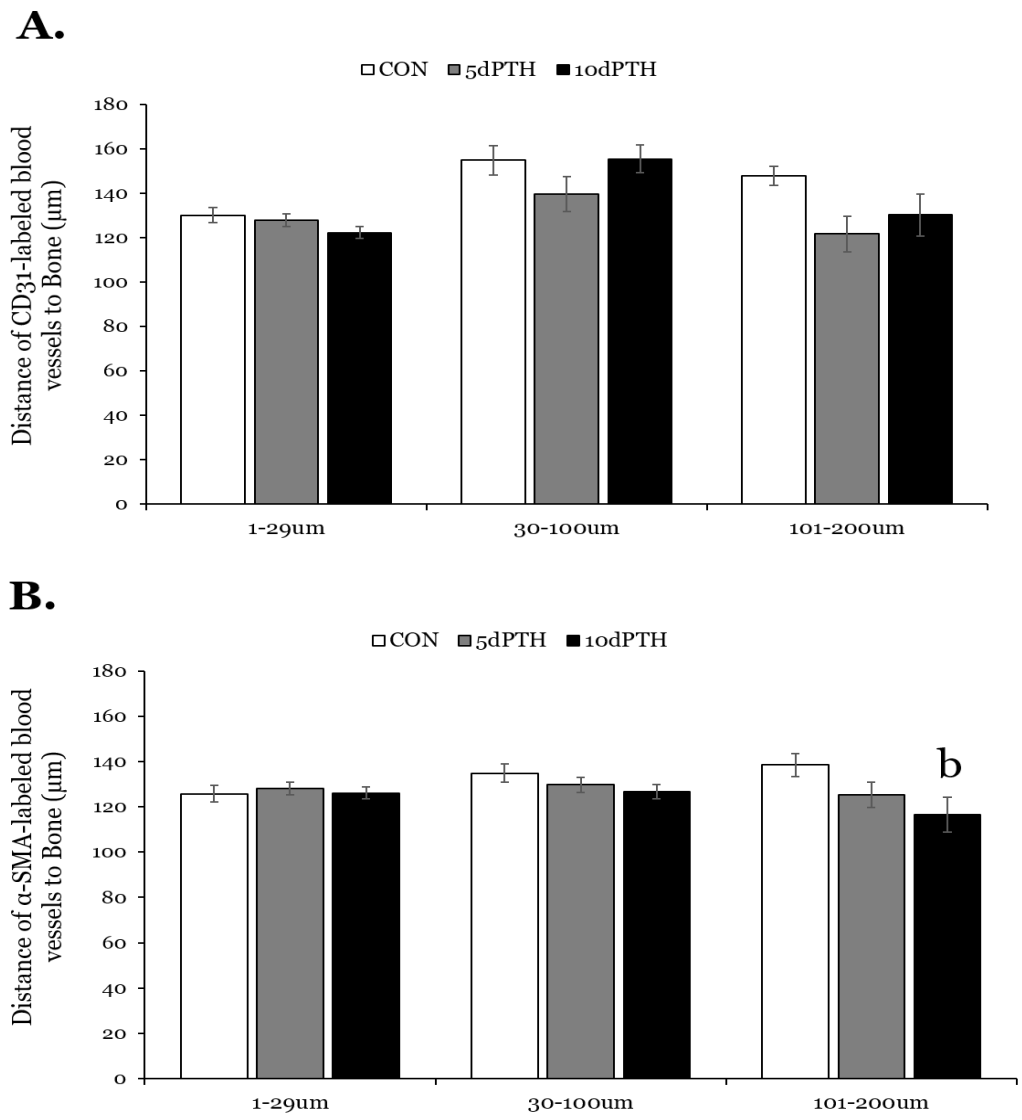

Figure 4.3. The distance of CD31- and αSMA-labeled bone marrow blood vessels from trabecular bone following 5- and 10-days of intermittent PTH 1-34 administration. A) Distances between 1-29µm, 30-100µm 101-200µm CD31-labeled bone marrow blood vessels from trabecular bone did not differ according to treatment. B) Distances between 1-29µm, 30-100µm 101-200µm αSMA-

labeled bone marrow blood vessels from trabecular bone did not differ according to treatment Values are means ± S.E. [b]p=0.076 vs. CON.

4.4.5 Distances between MMP-9, Bone Marrow Blood Vessels, and Trabecular Bone Surfaces

There were no significant main effects for age (Table 4.3) or treatment (Table 4.4) observed for distances of MMP-9 to CD31- and αSMA-labeled bone marrow blood vessels, and trabecular bone surface. Table 4.4 demonstrates that 10 days of intermittent PTH administration tended (p=0.096) to reduce the distance between MMP-9 and 1-29μm CD31-labeled bone blood vessels vs. CON.

Table 4.3. The main effect of age on the distance of MMP-9 from CD31- and αSMA-labeled bone marrow blood vessels and trabecular bone.

CD31	**Mature**	**Middle-Aged**
MMP-9 from 1-29μm	70.6 ± 1.5 μm	65.8 ± 2.9 μm
MMP-9 from 30-100μm	96.3 ± 1.5 μm	95.3 ± 3.1 μm
MMP-9 from 101-200μm	99.6 ± 5.0 μm	100.1 ± 3.8 μm
α-SMA	**Mature**	**Middle-Aged**
MMP-9 from 1-29μm	88.0 ± 1.4 μm	90.7 ± 1.6 μm
MMP-9 from 30-100μm	110.3 ± 0.9 μm	109.8 ± 1.2 μm
MMP-9 from 101-200μm	108.8 ± 1.6 μm	111.7 ± 1.1 μm
MMP-9 from Bone	130.6 ± 2.7 μm	123.0 ± 3.7 μm

Values represent Means ± S.E.
1-29μm, 30-100μm, and 101-200μm represents vessel diameter.

Table 4.4. The main effect of treatment on the distance of MMP-9 from CD31- and αSMA-labeled bone marrow blood vessels and trabecular bone.

CD31	**CON**	**5dPTH**	**10dPTH**
MMP-9 from 1-29μm	72.1 ± 2.8 μm	68.6 ± 2.6 μm	63.6 ± 2.8 μm [a]
MMP-9 from 30-100μm	93.2 ± 3.0 μm	97.6 ± 3.3 μm	96.6 ± 2.0 μm
MMP-9 from 101-200μm	100.9 ± 6.1 μm	100.8 ± 6.8 μm	98.0 ± 3.5 μm
α-SMA	**CON**	**5dPTH**	**10dPTH**
MMP-9 from 1-29μm	87.5 ± 2.0 μm	88.0 ± 1.6 μm	92.7 ± 1.5 μm
MMP-9 from 30-100μm	108.1 ± 1.1 μm	110.5 ± 1.4 μm	111.6 ± 1.2 μm
MMP-9 from 101-200μm	110.0 ± 1.6 μm	108.9 ± 1.7 μm	112.0 ± 1.7 μm
MMP-9 – Bone	130.8 ± 3.4 μm	127.1 ± 4.0 μm	122.5 ± 4.4 μm

Values represent Means ± S.E. [a]p=0.096 vs. CON.
1-29μm, 30-100μm, and 101-200μm represents vessel diameter.

4.4.6 MMP-9 Localization in Relation to Bone Marrow Blood Vessels and Trabecular Bone

To ascertain whether MMP-9 is secreted from trabecular bone or the bone marrow blood vessels, the CD31- and αSMA-labeled bone marrow blood vessels were pooled and analyzed according to diameter (1-29μm, 30-100μm, and 101-200μm). Figure 4.4 shows the distance of MMP-9 in relation to bone marrow blood vessels and trabecular bone. Contrary to our hypothesis, MMP-9 was localized closest ($p<0.05$) to the 1-29μm bone marrow blood vessel and localized farthest ($p<0.05$) from trabecular bone. The localization of MMP-9 was also further away ($p<0.05$) from bone marrow blood vessels 30-100μm and 101-200μm in comparison to the 1-29μm blood vessels.

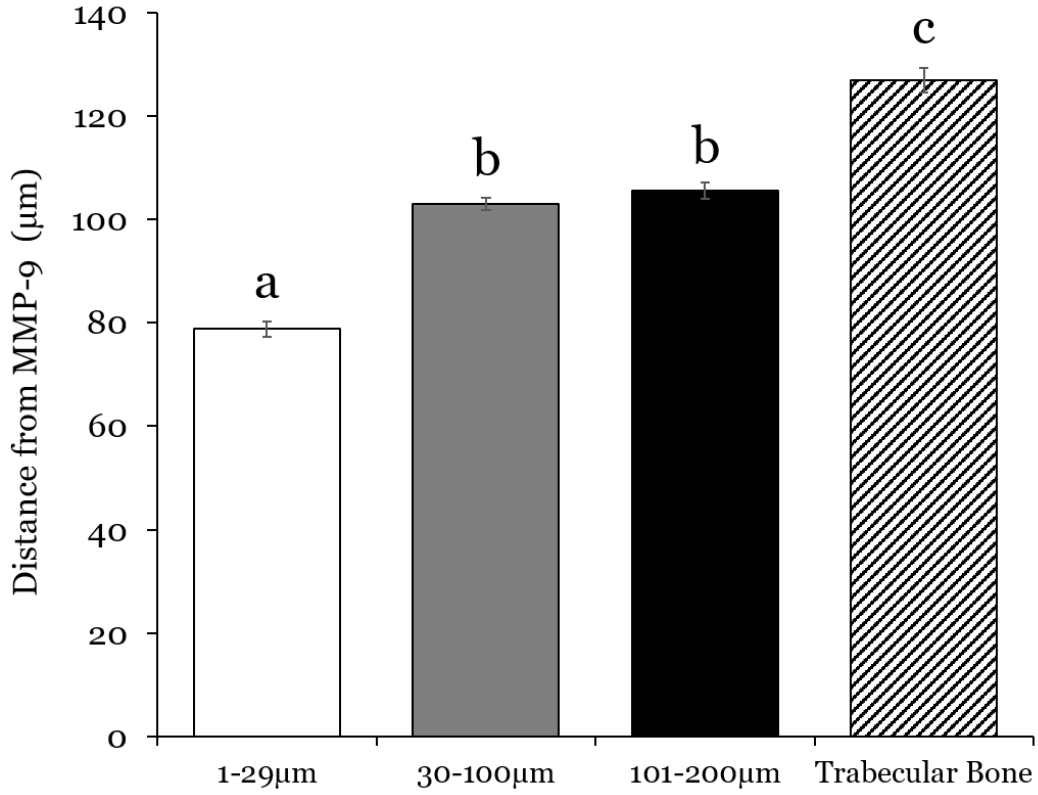

Figure 4.4. Comparison of MMP-9 localization in relation to bone marrow blood vessels (1-29µm, 30-100µm 101-200µm in diameter) and trabecular bone. Values are means ± S.E. [a]$p<0.05$ vs. 30-100µm, 101-200µm, and trabecular bone; [b]$p<0.05$ vs. 1-29µm and trabecular bone; [c]$p<0.05$ vs. 1-29µm, 30-100µm, and 101-200µm.

4.4.7 Bone Microarchitecture and Bone Static and Dynamic Properties

Table 4.5 presents data for bone microarchitecture and bone static and dynamic properties. As anticipated, no main effects for age or treatment, nor any significant interactions were observed.

Table 5. Bone microarchitecture and bone static and dynamic properties.

	Mature CON	Mature 5dPTH	Mature 10dPTH	Middle-Aged CON	Middle-Aged 5dPTH	Middle-Aged 10dPTH
Bone Microarchitecture						
BV/TV (%)	2.6±0.7	3.3±0.3	4.0±0.8	3.0±0.9	2.4±0.6	3.1±0.3
Tb.Th (μm)	22±2	22±1	23±1	22±2	19±1	21±4
Tb.N /mm^2	1.1±0.3	1.5±0.2	1.9±0.5	1.3±0.3	1.2±0.3	1.2±0.3
Tb.Sp (μm)	2472± 1061	718± 80	885± 247	1386± 403	1327± 402	2087± 898
Bone Static Properties						
Osteoblast Activity						
OS/BS (%)	4.0±0.9	4.4±1.2	7.4±1.4	6.0±1.5	4.5±0.7	4.7±1.5
Ob.S/BS (%)	1.8±0.4	1.9±0.3	1.9±0.2	1.8±0.5	2.2±0.5	1.8±0.2
Osteoclast Activity						
Oc.S/BS (%)	1.6±0.2	1.8±0.3	1.7±0.4	2.1±0.4	2.3±0.5	1.2±0.4
Bone Dynamic Properties						
MAR (μm/day)	0.16±0.03	0.20±0.03	0.16±0.02	0.19±0.03	0.19±0.03	0.17±0.03
sLS/BS (%)	2.50±0.31	2.39±0.31	2.55±0.15	2.02±0.23	2.07±0.22	2.34±0.20
dLS/BS (%)	0.68±0.14	0.92±0.14	1.01±0.18	0.80±0.11	0.92±0.17	0.96±0.52
MS/BS (%)	1.92±0.29	2.11±0.25	0.29±0.24	1.81±0.21	1.95±0.25	2.13±0.23
BFR/BS (μm^3/ μm^2/day)	0.0032± 0.0006	0.0047± 0.0011	0.0039± 0.0007	0.0035± 0.0006	0.0039± 0.0008	0.0037± 0.0008

Values represent Means ± S.E.

4.5 Discussion

The principal outcomes of this study demonstrate that 10 days of intermittent PTH 1-34 administration augmented the number of capillaries (i.e., 1-29μm diameter) (Figure 4.1). Thus, angiogenesis occurred prior to changes in trabecular bone microarchitecture and bone static and dynamic properties (Table 4.5). Furthermore, MMP-9 secretion was not augmented prior to 10 days of intermittent PTH administration (Figure 4.2) and was localized closest to capillaries in comparison to the larger sized vessels and trabecular bone (Figure 4.4).

Previous investigations demonstrated improved bone vascular function with intermittent PTH administration [4, 5, 10]. For example, endothelium-dependent vasodilator capacity of the femoral PNA was improved following 2 weeks of intermittent PTH 1-84 administration in young Wistar rats [5] and following 2 weeks of intermittent PTH 1-34 administration in old Fischer-344 rats [10]. However, discrepancies exist as to whether intermittent PTH administration enhances bone blood vessel number. For instance, lower vascular density was observed in the proximal tibia following 15- and 30-days of intermittent PTH 1-84 administration in Wistar rats [3]; however, vascular density was higher in tibiae following 14 days of intermittent PTH 1-84 administration in C57BL/6J mice [6]. Consistent with the previous mouse data [6], angiogenesis was induced with PTH treatment and occurred more rapidly than previously reported. In addition, vessels <100μm in diameter were most effected by PTH, coinciding with reports of

angiogenesis in blood vessels <90μm and <105μm in diameter following intermittent PTH 1-34 administration for 21 days and 28 days, respectively, in a mouse model of bone healing [23]. Similarly, bone angiogenesis was reported following 2 weeks of intermittent PTH administration in mice, which coincided with increased trabecular thickness and bone formation rate, decreased osteoclast activity but no change in bone volume [6]. The discrepancies in findings between the rat and mouse data may represent different physiological responses between the two species.

Similar to the data presented by Roche et al. [6], bone microarchitecture and bone static and dynamic properties were unaltered in the current study. Data from both studies indicate that changes in bone cellular activity with intermittent PTH administration occurs later than 10 days of treatment but are significantly altered by day 14. This outcome was anticipated due to the relatively short duration (5- and 10-days) of intermittent PTH administration. In accord, other studies have documented rapid alterations in the bone vascular system with PTH [6, 7]. For example, blood flow to the mouse hindlimb was augmented following a bolus dose of PTH 1-34, whereas it takes at least 2 week to observe alterations in bone [3, 5, 7]. In addition, 14 days of intermittent PTH 1-84 administration not only augmented bone blood vessels density by 16% and vessels area by 48%, but also increased bone perfusion by 27% and decreased bone vascular resistance by 23% [6].

Bone angiogenesis occurred rapidly in this investigation; i.e., bone vascular density was augmented 32% to 63% by 10 days of treatment. In addition, the current study reveals a possible association between intermittent PTH 1-34 administration and MMP-9-mediated angiogenesis in mice. Since MMP-9 plays a pivotal role in extracellular remodeling, which is crucial for vascular outgrowth and endothelial progenitor cell migration, it may promote angiogenesis and neovascularization [13, 14]. The role of MMP-9 in angiogenesis is initiated by vascular extracellular matrix degradation which facilitates endothelial cells invasion and outgrowth [24] and further stimulate the release of vascular endothelial growth factor [13]. For example, ischemic collateral blood flow was ~39% lower in MMP-9 deficient vs. wild-type mice, which coincided with reduced capillary density in the MMP-9 knockout animals [13]. Similarly, MMP-9 stimulated the migration of umbilical cord blood CD34+ cells [19], which plays an important role in angiogenesis [25, 26]. Similar to the current investigation, intermittent PTH administration increased the expression of MMP-9 in chondrocytes [27], in the rat long bone, and in bone tissue cultures [15]. For example, 5 days of intermittent PTH administration upregulated protein content of MMP-9 in mature osteoblasts and osteocytes from the tibial metaphysis and in cultures of osteoblasts and osteocytes [15]. Even though the total area of secreted MMP-9 was unaltered, the current study demonstrated increased ($p<0.05$) MMP-9 density following 10 days of intermittent PTH 1-34 administration in the distal

femoral metaphysis. Thus, increased secretion of MMP-9 with intermittent PTH administration may contribute to bone angiogenesis.

In addition to its role in matrix degradation, MMP-9 is involved in cellular migration and homing [19, 20]. In a previous investigation, intermittent PTH administration relocated small bone marrow blood vessels closer to osteoid seams [3]. Further, bone vascular density was lower in the PTH-treated vs. vehicle-treated rats [3]. Unfortunately, MMP-9 secretion was not measured in that investigation [3]; thus, the role of MMP-9 in blood vessel relocation could not be ascertained. MMP-9 density was also increased in this current investigation following 10 days of PTH administration. However, there was no differences in blood vessel distance from bone. Unfortunately, due to methodology limitations, the current investigation was unable to distinguish quiescent bone surfaces from active bone forming sites, as demonstrated in a previous study [3]. Thus, the spatial relationship between bone marrow blood vessels and osteoid seams was not measurable.

In the current investigation, I hypothesized that MMP-9 would be secreted from bone tissue, allowing for the attraction of bone marrow blood vessels closer to trabecular bone with PTH treatment. However, MMP-9 was spatially closer to the smallest blood vessels (<29μm) and further away from trabecular bone. MMP-9 secretion is not limited to osteoclasts, osteoblasts and osteocytes [15, 28], but are produced and released from other cells such as vascular endothelial cells [29]. Thus,

given its closer proximity to capillaries (<29μm), it is possible that MMP-9 was secreted from the vascular endothelial cells.

In conclusion, current results demonstrate that the density of the smallest blood vessels (1-29μm) was augmented following 10 days of intermittent PTH 1-34 administration; i.e., at a time point when trabecular bone was not altered. In addition, MMP-9 secretion was enhanced by day 10, which presumably stimulated angiogenesis. Further, MMP-9 was localized closest to the smallest blood vessels, while localized farthest away from trabecular bone. Given the rapid response in bone angiogenesis (i.e., within 10 days), this investigation highlights the impact of intermittent PTH administration on the bone vascular network.

4.6 Conflicts of Interest

Seungyong Lee and Rhonda D. Prisby declare that they have no conflicts of interest.

4.7 Acknowledgements

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Chapter 5

Future Directions

This dissertation examined the age-related effects of short-term intermittent PTH 1-34 administration on bone marrow blood vessel ossification, spatial relocation of bone marrow blood vessels, angiogenesis, and MMP-9 in mice. Additionally, bone microarchitecture and bone static and dynamic properties were assessed. While much information was ascertained, more questions were raised regarding the duration of intermittent PTH administration, the spatial location of bone marrow blood vessels in regards bone forming sites (i.e., osteoid seams), and bone blood flow measurements.

The findings on bone marrow blood vessel ossification demonstrated augmented ossified vessel thickness in the Middle-Aged vs. Mature mice. In addition, ossified vessel volume tended to increase as a function of advancing age and ossified vessel thickness tended to increase following 10-day of intermittent PTH administration. These tendencies suggest that the duration of PTH administration in the current dissertation was too short to observe significant differences. It seems reasonable to assume that longer duration studies would yield significant changes in bone marrow blood vessel ossification.

In Specific Aim 2, examined bone vascular density and bone marrow blood vessel relocation. Technical limitations in methodology prevented the distinction between quiescent bone surfaces vs. sites of bone formation (i.e., osteoid). Thus,

future studies may include assessing vascular density and bone marrow blood vessel location in relation to quiescent bone surfaces separate from osteoid seams.

Further, while able to determine MMP-9 localization in relation to trabecular bone and bone marrow blood vessels, it was not possible to assess which cells were producing and releasing MMP-9 with intermittent PTH administration. Thus, future studies utilizing cultured cells exposed to varying concentration of PTH would allow for this determination. For example, cell culture media could be assayed for MMP-9 following exposure of endothelial, smooth muscle and MC3T3-E1 (i.e., pre-osteoblasts) cells to PTH.

In addition, identification of genome regions linked to vascular calcification and ossification may be required. Thus, the discovery of the gene mutation that may regulate vascular extracellular matrix calcium deposition and phosphate production in the bone marrow microenvironment would be invaluable.

Lastly, the assessment of in vivo bone blood flow in conscious mice following intermittent PTH administration would be invaluable. While technically challenging, these data would surpass the bone perfusion data currently available in the literature and perhaps allow for the definitive conclusion as to whether intermittent PTH administration augments skeletal blood flow.

Appendix A

Reproducibility of Distance Measurements between Bone Marrow Blood Vessels and Trabecular Bone

Appendix A. Reproducibility of distance measurements between CD31- and α-SMA-labeled bone marrow blood vessels and trabecular bone in Mature mice.

Group	Variable	Day 1	Day 2	Day 3	*F*	*P*
Mature CON	CD31 (1-29μm)	161.3 ± 7.3 (μm)	161.3 ± 7.3 (μm)	161.3 ± 7.3 (μm)	0.00	1.00
	α-SMA (1-29μm)	105.7 ± 17.8 (μm)	105.7 ± 17.8 (μm)	105.7 ± 17.8 (μm)	0.00	1.00
Mature 5dPTH	CD31 (1-29μm)	159.8 ± 21.1 (μm)	159.8 ± 21.1 (μm)	159.8 ± 21.1 (μm)	0.00	1.00
	α-SMA (1-29μm)	159.0 ± 22.8 (μm)	159.0 ± 22.8 (μm)	159.0 ± 22.8 (μm)	0.00	1.00
Mature 10dPTH	CD31 (1-29μm)	199.5 ± 21.2 (μm)	199.5 ± 21.2 (μm)	199.5 ± 21.2 (μm)	0.00	1.00
	α-SMA (1-29μm)	189.2 ± 30.3 (μm)	189.2 ± 30.3 (μm)	189.2 ± 30.3 (μm)	0.00	1.00
Middle-Aged CON	CD31 (1-29μm)	134.2 ± 8.1 (μm)	134.2 ± 8.1 (μm)	134.2 ± 8.1 (μm)	0.00	1.00
	α-SMA (1-29μm)	104.5 ± 15.8 (μm)	104.5 ± 15.8 (μm)	104.5 ± 15.8 (μm)	0.00	1.00
Middle-Aged 5dPTH	CD31 (1-29μm)	199.9 ± 27.8 (μm)	199.9 ± 27.8 (μm)	199.9 ± 27.8 (μm)	0.00	1.00
	α-SMA (1-29μm)	106.4 ± 29.0 (μm)	106.4 ± 29.0 (μm)	106.4 ± 29.0 (μm)	0.00	1.00
Middle-Aged 10dPTH	CD31 (1-29μm)	69.0 ± 10.5 (μm)	69.0 ± 10.5 (μm)	69.0 ± 10.5 (μm)	0.00	1.00
	α-SMA (1-29μm)	175.1 ± 28.9 (μm)	175.1 ± 28.9 (μm)	175.1 ± 28.9 (μm)	0.00	1.00

Values represent Means ± S.E. One-way ANOVA with repeated measure was used.

Biographical Information

Seungyong Lee, Ph.D., is a native of South Korea and researcher whose career in exercise and applied physiology extends over eight years. He received a bachelor's degree in Physical Education from Dankook University in 2006, a master's degree in Exercise Physiology from University of Kentucky in 2013, and Ph.D. in Kinesiology from the University of Texas at Arlington in 2018. His research interests is focused on the interactions between blood vessels and bone in rodent models. He examined the effects of intermittent PTH administration on the bone vascular system, bone microarchitecture, and bone static and dynamic properties as a function of advancing age in mice.

During his tenure as a Ph.D. student, Seungyong has been awarded several fellowships and awards including a Summer Dissertation Fellowship 2018 from the University of Texas at Arlington, a Cardiovascular Section Research Recognition Award from the American Physiological Society, a Dean's Award for Outstanding Poster Presentation at the 2017 Annual Celebration of Excellence by Students Symposium at the University of Texas at Arlington, and a Caroline tum Suden / Frances Hellebrandt Professional Opportunity Award in 2016 from the American Physiological Society. Additionally, he has published two manuscripts and submitted two first-author manuscripts related to his dissertation. He will be a co-author on three additional manuscripts currently under review and a co-author on multiple manuscripts to be submitted in the future. To date, he had seven first-

author and three co-author published abstracts presented at national and international conferences. He will continue his training as a post-doctoral fellow in the Department of Pathology at Johns Hopkins Medicine, where he will investigate the influence of pericytes and nerve innervation on bone healing and regeneration. Currently, he is a member of the Bone Vascular and Microcirculatory Society (BVMS), American Society for Bone Mineral Research (ASBMR), American Physiological Society (APS), and American College of Sports Medicine (ACSM).