

Mechanical, hormonal and metabolic influences on blood vessels, blood flow and bone

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Abstract

Bone tissue is highly vascularized due to the various roles bone blood vessels play in bone and bone marrow function. For example, the vascular system is critical for bone development, maintenance and repair and provides O₂, nutrients, waste elimination, systemic hormones and precursor cells for bone remodeling. Further, bone blood vessels serve as egress and ingress routes for blood and immune cells to and from the bone marrow. It is becoming increasingly clear that the vascular and skeletal systems are intimately linked in metabolic regulation and physiological and pathological processes. This review examines how agents such as mechanical loading, parathyroid hormone, estrogen, vitamin D and calcitonin, all considered anabolic for bone, have tremendous impacts on the bone vasculature. In fact, these agents influence bone blood vessels prior to influencing bone. Further, data reveal strong associations between vasodilator capacity of bone blood vessels and trabecular bone volume, and poor associations between estrogen status and uterine mass and trabecular bone volume. Additionally, this review highlights the importance of the bone microcirculation, particularly the vascular endothelium and NO-mediated signaling, in the regulation of bone blood flow, bone interstitial fluid flow and pressure and the paracrine signaling of bone cells. Finally, the vascular endothelium as a mediator of bone health and disease is considered.

Key Words

- ▶ bone formation and resorption
- ▶ angiogenesis
- ▶ endothelium
- ▶ estrogen
- ▶ exercise

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Introduction

The vascular system is indispensable for the health and longevity of all biological tissues and organisms (North & Sinclair 2012). The demand for O₂, nutrients and waste elimination requires the foremost development of the vascular system during embryogenesis (Stocum 2006). In addition, regeneration of the vascular system is critical and ubiquitous for repair of all tissues (Stocum 2006). Therefore, a major requisite for biological activity is the vascular supply (Burkhardt *et al.* 1987). For years, bone biologists have focused their attention on the cellular and molecular functions of the skeleton in health and disease while often underappreciating the bone vascular network

(i.e., the organ system that enables the skeleton to form and function). As poignantly stated by a pioneer in bone biology, 'How can the methods of cell and molecular biology be deployed to study a biologic phenomenon that is manifested only in the intact organism?' (Parfitt 1994). While speaking of bone remodeling in the context of the activities of the bone basic multicellular unit (Parfitt 1994), this statement can be equally applied to the interactive and regulatory nature among the vascular system, bone and bone marrow.

Although gaining more attention in recent years, the importance of the vascular supply to the skeleton

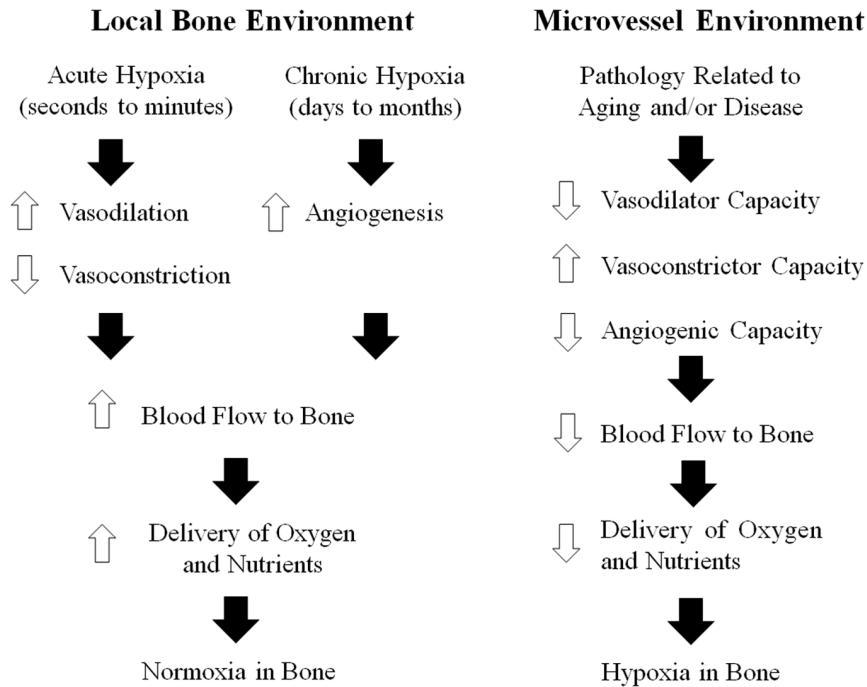
has not undergone historical indifference (Lacey 1929, Brookes 1957, Brookes & Revell 1998). Another pioneer investigator stated that 'intraosseous blood flow gives life to bone, just as intracerebral blood flow gives life to the brain and coronary blood flow life to the heart' (Laroche 2002). Additionally, Brookes and Lloyd (1961) theorized that bone formation occurs only in relation to blood vessels, capillaries and sinusoids and that the structural development of the vascular pattern of mammalian bone marrow precedes and contributes significantly to the arrangement of bone trabeculae (Brookes & Lloyd 1961).

Besides providing O₂ and other essential nutrients critical for bone metabolism, bone blood vessels deliver systemic hormones and precursor cells for bone remodeling (Fujikawa *et al.* 1996, Eghbali-Fatourehchi *et al.* 2005), are integral components of bone basic multicellular units (Parfitt 1994, 1998), participate in hematopoiesis (Arai & Suda 2007) and are egress and ingress routes for blood and immune cells to and from the bone marrow (Mazo & von Andrian 1999, Nilsson *et al.* 2001, Martin *et al.* 2003). Further, bone development and maintenance are dependent upon the bone vascular network. For example, vascular ingrowth is necessary for endochondral and intramembranous bone formation, as reviewed by Kanczler and Oreffo (2008). Eventually, the skeletal system develops into an exceedingly vascularized tissue (Doll 2005). Throughout the longevity of the organism, the vascular supply is a constant necessity for the healthy maintenance of the skeleton.

The human skeleton contains over 200 bones, which are constantly undergoing modeling and remodeling to maintain bone strength (Clarke 2008). Modeling occurs as a result of the adaptation of bone in response to daily physiological stimuli, can occur via the independent activities of osteoblasts and osteoclasts (Clarke 2008) and declines with adulthood (Kobayashi *et al.* 2003). The maintenance of bone strength and mineral homeostasis occurs via remodeling, whereby localized pockets of old or damaged bone is renewed (Clarke 2008). Remodeling of bone occurs with bone basic multicellular units for cortical bone (Frost 1983b) and bone remodeling compartments for trabecular bone (Hauge *et al.* 2001). These units/compartments comprise bone-resorbing osteoclasts, bone-forming osteoblasts and a vascular capillary (Parfitt 1994, 1998, Melsen *et al.* 1995). The capillary of the bone basic multicellular unit is theorized to provide nutrients, O₂ and precursor cells to sites of bone remodeling. By default, the enhanced metabolism associated with bone modeling and remodeling augments blood flow.

Augmenting (or diminishing) tissue blood flow occurs by (1) the creation of new blood vessels via angiogenesis or arteriogenesis (or vascular rarefaction) and/or (2) by vasodilation (or vasoconstriction) of resistance arteries. Resistance arteries (i.e., arterioles and nutrient arteries) provide immediate regulation of tissue blood flow by altering blood vessel diameter with vascular smooth muscle contraction and relaxation, as reviewed by Hill and coworkers (Hill *et al.* 2001). Vascular smooth muscle contraction and relaxation are governed by systemic hormones, neural influences and locally produced factors designed to maintain tissue perfusion and blood pressure (Hill *et al.* 2001). There are several systemic hormones that control bone metabolism and the vasomotor activity of blood vessels. Neural control of the vasculature comes in the form of neurotransmitters and neuropeptides released by the nervous system (Bjurholm *et al.* 1988a,b, Hill & Elde 1991). Further, autocrine and paracrine factors are produced and released locally as a result of metabolism, perfusion pressure or shear stress acting on the vascular wall (Hill *et al.* 2001) and on bone cells as a result of interstitial fluid flow (Johnson *et al.* 1996).

Local environmental factors in bone modify blood vessel activity. In response to acute tissue hypoxia, local blood vessels increase tissue perfusion via enhanced vasodilator or diminished vasoconstrictor mechanisms. Following periods of chronic hypoxia, whereby vasomotor mechanisms to augment tissue perfusion are inadequate, enhanced blood flow can also result from angiogenesis (i.e., the formation of new from existing blood vessels). Another aspect to consider is the tissue hypoxia resulting from impaired vasomotor responses of local blood vessels due to pathologies within the vascular system. Figure 1 depicts the sequence of events following periods of acute or chronic hypoxia in bone. Further, the figure illustrates how dysfunction of the bone vascular network can lead to hypoxia within bone. While not as clinically immediate as occlusion of blood flow to the brain or heart, reduced or impeded blood flow to the skeleton manifests into several sub-clinical and clinical pathologies of bone and bone marrow. On the contrary, adequate delivery of nutrients, O₂, hormones, growth factors, cytokines and cells on a beat-to-beat basis allows for the proper functioning of bone and bone marrow on a daily basis. This review presents evidence of how the vascular system contributes to bone health and presents information on bone anabolic agents that also control bone blood vessels and bone blood flow. Further, this review may guide the reader into a systemic, integrative approach to bone metabolism in health and disease.

**Figure 1**

Hypoxia in bone. Acute hypoxia (seconds to minutes) will elicit rapid vasomotor responses (i.e., vasodilation and/or vasoconstriction) from bone arteries and arterioles to augmented blood flow and normalize the partial pressure of oxygen in the local environment. Chronic hypoxia (days to months) results from an inability of the arteries and arterioles to adequately augment tissue blood flow and oxygen. Under these circumstances, angiogenesis will ensue in order to meet the needs of the tissue. In regards to how the vascular system may impact bone health, dysfunction of the bone vasculature with advancing age and/or disease cause imbalances in arteriolar vasodilation and vasoconstriction and limit angiogenic capability. The result is diminished blood flow and oxygen delivery to bone. Under these circumstances, the etiology of hypoxia lies with the bone vascular network as opposed to with the enhanced metabolic activity of bone.

The microcirculation of bone

Similar to other circulatory beds, the circulation of bone comprises of (1) afferent vessels, (2) a microvascular network and (3) efferent vessels (Brookes & Revell 1998, McCarthy 2006). Capillaries and sinusoids are the sites of ionic exchange between the blood and bone and bone marrow cells (Brookes & Revell 1998). The arteries consist of the (1) metaphyseal, (2) epiphyseal, (3) principal nutrient artery (PNA) and (4) periosteal vessels (Brookes & Revell 1998). The metaphyseal, epiphyseal and periosteal vascular networks supply the regions of skeletal tissue from whence they obtain their names and the PNA supplies the diaphyseal marrow and inner 2/3rds of the diaphyseal cortex (Brookes & Revell 1998).

The PNA enters the cortex and branches into an ascending and descending limb, which runs down the center and traverses the length of the shaft, as reviewed by McCarthy (2006). Branches of the PNA spread out toward the endosteum and eventually ramify into sinuses or sinusoids, as reviewed by Gurkan and Akkus (2008). The collection of these sinuses or sinusoids journey back to the center of the shaft and into the principal nutrient vein, juxtaposed with the ascending and descending PNA, and exits the cortex through the principal nutrient foramen (Brookes & Revell 1998, Gurkan & Akkus 2008). Besides the principal nutrient vein, other routes of egress of blood include the epiphyseal, metaphyseal and cortical-periosteal veins (Brookes & Revell 1998).

Thus, the endosteal surface is replete with vessels and sinuses (Gurkan & Akkus 2008), which provides enhanced surface area for exchange and slower blood flow rates at these locations (Gurkan & Akkus 2008).

The vascularization of flat bones is similar to that in long bones; i.e., there is (1) a PNA, (2) numerous, smaller metaphyseal nutrients and (3) a profuse sinusoidal network in the marrow space with continued hematopoiesis (Brookes & Revell 1998). Similar to long bones, flat bones also have a periosteal vascular network, which provides the majority of perfusion (Brookes & Revell 1998). The importance of the periosteal network in membranous bones differs from that observed in young long bones, whereby the blood supply from this periosteal network is minimal. However, it is similar to aged long bones where increased reliance from the periosteal input is observed (Brookes & Revell 1998). Understanding the anatomical layout and functional properties of the bone circulation in relation to the cells of bone and bone marrow and elucidating the age- and disease-related alterations in vascular function under these contexts are important aspects to consider when examining the biological function of the skeletal system as a whole.

Ten-to-eleven and five percent of cardiac output in rats and humans, respectively, is dedicated to skeletal perfusion (Wootton *et al.* 1976, 1978, Charkes *et al.* 1979, Gross *et al.* 1979), such that bone blood flow is similar to resting blood flow to some skeletal muscles (Delp *et al.* 1999). Blood flow data collected via the

injection of radiolabelled microspheres into conscious rodents support this contention (Prisby *et al.* 2007). Femoral bone blood flows in young rats descended in the following manner: diaphyseal marrow, distal metaphysis, proximal metaphysis and diaphyseal cortex (Prisby *et al.* 2007). Regional blood flow differences in the diaphyseal marrow, distal metaphysis and proximal metaphysis were significantly diminished in old rats, while blood flow to the diaphyseal cortex remained unchanged (Prisby *et al.* 2007). Table 1 highlights the comparison between resting blood flows in the femur and skeletal muscles in rats (Delp *et al.* 1999, Prisby *et al.* 2007).

The attenuated femoral bone blood flow with advanced age corresponded with reduced endothelium-dependent vasodilation of the femoral PNA (Prisby *et al.* 2007). Blood flow in long bones are regionally dependent; i.e., hematopoietic marrow and the diaphyseal cortex have the highest and lowest blood flows, respectively (Davis & Wood 1997, Prisby *et al.* 2007) and regional differences in blood flow relate to the varied metabolic activities in the separate bone compartments (Brookes 1967). Protein synthesis is high during hematopoiesis and, therefore, high blood flows are expected in the marrow (Brookes 1967). Further, bone synthesis also requires considerable amounts of energy (Brookes 1967) and ionic exchange is augmented during bone growth (Leblond *et al.* 1950, Amprino & Murotti 1964) and coincides with higher blood flows (Brookes 1967). Thus, regional blood flows may serve as indicators of bone turnover and metabolism, and alterations in bone blood flow is a key influence on local bone accrual (Brookes 1967).

Mechanical loading

Wolff's Law characterizes how bone adapts to functionally withstand its mechanical environment (Wolff 1986). Conditions of chronically increased or decreased mechanical loading will augment (Westerlind *et al.* 1997, Dominguez *et al.* 2010) and diminish (Colleran *et al.* 2000, Prisby *et al.* 2015) bone mass, respectively. To initiate bone adaptation via mechanical loading, bone tissue must experience minimum strains of at least 800–2000 μ strain (Frost 1983a, Westerlind *et al.* 1997, Frost 2001). However, this threshold may be lower for some skeletal sites (Hsieh *et al.* 2001). Interestingly, *in vivo* experiments that altered intramedullary pressure and/or fluid flow without eliciting bone matrix strain have highlighted that bone accrual does occur (Kelly & Bronk 1990, Revell & Brookes 1994, Kwon *et al.* 2010, Qin *et al.* 2003). Thus, mechanical loading *per se* is not the direct stimulus for bone remodeling.

Bone tissue is highly fluidic and interstitial fluid flow is involved in the mechanically induced activation of bone cells (Cowin & Cardoso 2015). Mechanical loading of the skeleton creates pressure gradients that expels interstitial fluid from areas of bone that are compressed and reuptakes the interstitial fluid once the compression ends (Piekarski & Munro 1977). Since bone is a porous tissue composed of a collagen–hydroxapatite matrix with a lacunar–canalicular network (Beno *et al.* 2006), compression and tension serves to transport interstitial fluid throughout bone (Piekarski & Munro 1977). Leaky capillaries within the marrow cavity are the source of this

Table 1 Comparison of skeletal muscle and femoral bone regional blood flows in the rat.

Rat characteristic	Tissue	Blood flow (mL/min/100g)	Reference
Male Sprague–Dawley rats (399–564 g)	Soleus	95	Delp <i>et al.</i> (1999)
	Plantaris	16	
	Gastrocnemius		
	Red	54	
	Middle	18	
	White	10	
	Tibialis anterior		
Red	59		
White	25		
Male Fischer-344 rats (4–6 months, 341 g)	Extensor digitorum longus	16	Prisby <i>et al.</i> (2007)
	Femoral diaphyseal marrow	39	
	Distal femoral metaphysis	29	
	Proximal femoral metaphysis	26	
	Femoral diaphyseal cortex	13	
Male Fischer-344 rats (22–24 months, 411 g)	Femoral diaphyseal marrow	21	Prisby <i>et al.</i> (2007)
	Distal femoral metaphysis	20	
	Proximal femoral metaphysis	18	
	Femoral diaphyseal cortex	11	

Values represent means.

fluid flow (Montgomery *et al.* 1988), as these vessels are the final recipient of blood supplied from the nutrient arteries. Nutrient arteries also modulate bone intramedullary pressure through the circulatory supply (Lam *et al.* 2010). Shear forces acting upon bone cell surfaces, as a result of interstitial fluid flow, provide the stimulus for mechanical loading-induced activation of bone cells and based upon the stimulus (or lack thereof) activates bone anabolic or catabolic responses (Cowin & Cardoso 2015).

Bone cells react to the interstitial fluid flow and pressure in the bone marrow and the lacunar–canalicular network of bone, which creates shear stress along their surface membranes. Shear stress stimulates the release of factors such as nitric oxide (NO) and prostaglandins (e.g., PGE₂) by bone-derived cells and organ cultures (Johnson *et al.* 1996, McAllister & Frangos 1999, Zaman *et al.* 1999, McAllister *et al.* 2000). For example, under 12 dynes/cm² shear stress, the release of adenosine triphosphate (ATP), NO and PGE₂ from rat primary osteoblasts was significantly higher vs static control conditions (Li *et al.* 2016). These factors can modulate bone cellular activity. For example, NO aids in osteoblasts differentiation (Riancho *et al.* 1995, Hikiji *et al.* 1997) and inhibits bone resorption by osteoclasts (Kasten *et al.* 1994). Further, PGE₂ prevented bone loss associated with hind limb immobilization and augmented trabecular bone to values higher than age-matched, control animals (Akamine *et al.* 1992). In fact, augmented wall shear stress induced by fluid flow enhanced NO release from osteoblastic cells but mechanical strain did not elicit the same response (Smalt *et al.* 1997). Under *in vivo* conditions, unloaded turkey ulnae subjected to oscillatory intramedullary fluid pressure loading in the absence of bone matrix strain demonstrated bone accrual in the diaphysis (Qin *et al.* 2003). Delivery of oscillatory intramedullary fluid pressure at 3 Hz for 3 weeks without accompanying bone matrix deformation of the ulna, which generated intramedullary pressures between 50 and 90 mmHg, was shown to increase cross-sectional lumen area (+14%, statistically non-significant), arterial wall area (+46%) and wall thickness (+28%) of the nutrient artery (Lam *et al.* 2010). Bone adaptation under a similar fluid pressure loading protocol occurred at 4 weeks (Qin *et al.* 2003). Thus, the outward hypertrophic remodeling of the ulnar nutrient artery, which is consistent with chronically increased pressure and blood flow experienced by vessels (Langille 1995, Osol & Mandala 2009), preceded or occurred concomitantly with the initiation of bone adaptation (Lam *et al.* 2010).

When subjected to regular physical activity, bone adapts so that improvements in bone mineral density and

bone health are observed (Modlesky & Lewis 2002). It must be noted that alterations in hormonal and neural activity occur with exercise. Thus, the adaptations in bone and bone blood vessels with physical activity reflect these influences as well. However, given the context of this section of the article, these influences will not be addressed. So how may the bone vascular system contribute to the improvements in bone health associated with physical activity? During low- and moderate-intensity treadmill exercise in canines, mean arterial pressure increased ($P > 0.05$; 21% and 39%, respectively) and vascular resistances were augmented in the sternum, rib, ilium, inferior femoral epiphysis and femoral diaphysis (Gross *et al.* 1979). Blood flow to these various bones and bone regions was preserved, presumably as a result of augmented mean arterial pressure, despite the rise in bone vascular resistance (Gross *et al.* 1979). The ilium was the only bone to experience declines in blood flow with exercise and, overall, these data suggest that vasoconstriction occurred in the bone vasculature (Gross *et al.* 1979). In contrast, blood flow to various hind limb bones and bone regions was augmented with 5 min of treadmill exercise in sedentary young and old rats (Dominguez *et al.* 2010) and in young sedentary and exercise-trained rats (Stabley *et al.* 2014) vs resting conditions. Mean arterial pressure did not differ between the young sedentary and exercise-trained animals at rest and during exercise (Stabley *et al.* 2014). Only blood flow to the femoral diaphysis in the exercised-trained rats was significantly higher than the sedentary group (Stabley *et al.* 2014). In other words, the sedentary animals were able to augment regional bone blood flow during acute exercise to similar degrees as the exercise-trained rodents (Stabley *et al.* 2014).

Altering bone blood flow during acute exercise occurs immediately via changes in vasodilation and/or vasoconstriction of bone blood vessels. Following chronic exercise, changes in bone blood flow occur via modifications in the functional properties of bone blood vessels and/or angiogenesis. In addition to the aforementioned processes, a third mechanism by which to increase bone blood flow is through the exercise-induced augmentation in systemic blood pressure for a given bone vascular resistance. Age-related declines ($P = 0.06$) in bone volume (–22%) were demonstrated in old (22–24 months) vs young (4–6 months) rats (Prisby *et al.* 2007). Declines in bone volume corresponded with diminished endothelium-dependent vasodilation of the femoral PNA, reduced NO bioavailability for the PNA and reduced femoral bone blood flow vs the younger counterparts (Prisby *et al.* 2007). Following 10–12 weeks of treadmill

exercise training, bone volume and NO-mediated, vasodilator capacity of the femoral PNA were enhanced in both the young and old groups (Dominguez *et al.* 2010). Given the measured strain magnitudes of rat femora and measured strain during compression and tension of rat tibiae with treadmill running (Indrekvam *et al.* 1991, Rabkin *et al.* 2001), alterations in strain magnitude during this treadmill protocol (Dominguez *et al.* 2010) were presumably lower than the minimum threshold needed for bone adaptation (Frost 1983a, 2001, Westerlind *et al.* 1997). Thus, augmented femoral bone blood flow elicited by daily bouts of treadmill exercise served to enhance vasodilator function and presumably caused alterations in bone interstitial fluid flow and pressure as well (Dominguez *et al.* 2010). Additionally, when comparing hind limb bone blood flow between rest and an acute bout of treadmill exercise (5 min) in sedentary and exercise-trained (10–12 weeks of treadmill exercise) rats, exercise hyperemia occurred in 33% and 78% of the examined bone regions in the sedentary and exercised-trained animals, respectively (Stabley *et al.* 2014). The authors concluded that exercise training delivers blood to more regions of bone as opposed to enhancing blood flow capacity (Stabley *et al.* 2014).

In regards to angiogenesis as a means to augment blood flow to bone following chronic exercise training, blood vessel number in the tibial metaphysis of young rats was augmented by 19% vs sedentary animals following 2 weeks of treadmill exercise (Yao *et al.* 2004). Bone mineral density and bone volume were augmented following 4 and 5 weeks, respectively; i.e., 2–3 weeks subsequent to the rise in bone vascularity (Yao *et al.* 2004). Interestingly, when bone angiogenesis was inhibited with administration of an anti-vascular endothelial growth factor antibody in conjunction with treadmill exercise training, bone mineral density, bone volume and blood vessel number were comparable to the sedentary group (Yao *et al.* 2004); i.e., the inhibition of the vascular adaptation to exercise prohibited the adaptation to bone, even in the face of mechanical perturbation. Similarly, bone capillarity in aged, exercise-trained rats was augmented following 8 weeks of swimming in comparison to aged control animals (Viboolvorakul *et al.* 2009). The interesting aspect of this study is that the non-weight-bearing activity of swimming minimized the mechanical loads placed upon the skeleton in comparison to weight-bearing exercise such as treadmill running. While swimming, the skeleton would have only experienced the mechanical loads associated with muscle contraction; however, alterations in bone vascular density were still

observed. Unfortunately, no parameters were reported for potential alterations in bone (Viboolvorakul *et al.* 2009).

In conclusion, the vascular system provides blood supply to the skeleton, which delivers nutrients, O₂, precursor cells, etc. and removes wastes products produced as a result of metabolism. Through its regulation of interstitial fluid flow and pressure, bone blood vessels also stimulate the release of various factors from bone and bone marrow cells to regulate their activity. As a result of chronic mechanical perturbation, the vascular system can contribute to bone accrual by supplying larger areas of the skeleton with blood flow either through improved vasodilator capacity of bone blood vessels and/or via angiogenesis. Further, the adaptations in the vascular system may precede changes occurring in bone.

Parathyroid hormone and parathyroid hormone-related peptide

After production and release from the parathyroid glands, parathyroid hormone (PTH) 1–84 circulates systemically. Parathyroid hormone-related peptide (PTHrP) is produced locally and acts at the tissue level (Takahashi *et al.* 1995). The main role of PTH 1–84 is to maintain serum calcium homeostasis, and this occurs via bone resorption and calcium retention (Kousteni & Bilezikian 2008). Continual release of PTH 1–84 or administration of PTH 1–34 and PTHrP 1–36 caused declines in bone mineral density and bone formation and increased bone resorption (Hansen *et al.* 2010, Horwitz *et al.* 2011); however, when administered intermittently (e.g., one injection per day), PTH causes bone gain (Stewart *et al.* 2000, Prisby *et al.* 2011, 2013). Further, the anabolic actions of intermittent PTHrP administration have been explored (Amizuka *et al.* 1996, Stewart *et al.* 2000, Horwitz *et al.* 2003, Miao *et al.* 2005, de Castro *et al.* 2012) and are under clinical consideration (Leder *et al.* 2015).

The enhanced bone metabolism associated with PTH-induced bone accrual during intermittent administration must be accompanied by augmented bone blood flow. Thus, the role of the bone vascular network in facilitating bone accrual is of importance to understand. Charbon and coworkers were the first to demonstrate the vasodilator effects of PTH on the systemic vasculature (Charbon *et al.* 1968, Charbon 1969, Charbon & Hulstaert *et al.* 1974) and PTHrP is theorized to regulate vascular tone (Takahashi *et al.* 1995). Vasodilation of bone blood vessels occurs in the presence of PTH; e.g., vasodilation in isolated rat femoral PNAs was observed to increasing concentrations

of PTH 1–84, PTH 1–34 and PTHrP 1–34 (Benson *et al.* 2016). Additionally, bolus doses of PTH have produced a rise (Kapitola & Zák 2003, Gohin *et al.* 2016), a biphasic response (Boelkins *et al.* 1976) and no change (Driessens & Vanhoutte 1981) in blood flow to various hind limb bones. In regards to the chronic influences of intermittent PTH administration on bone blood vessels, the picture becomes more complex. As mentioned previously, blood flow can be augmented via the vasomotor activity of resistance vessels and via angiogenesis. Thus, PTH acts upon the functional and/or morphological properties of bone blood vessels.

Several reports in the literature have measured various bone vascular parameters following intermittent PTH administration to address these questions. Bone angiogenesis was not observed following 15 and 30 days of intermittent PTH 1–84 administration in young rats (Prisby *et al.* 2011). In fact, bone vessel density was lower in PTH-treated vs control animals (Prisby *et al.* 2011). Interestingly, the smallest bone marrow blood vessels in PTH-treated rats were closer to sites of new bone formation (Prisby *et al.* 2011), which is theorized to direct blood flow where needed the most and facilitate nutrient exchange between the vascular system and bone. Additionally, 15 days of intermittent PTH 1–84 administration enhanced endothelium-dependent vasodilation of the femoral PNA via an upregulation of NO signaling (Prisby *et al.* 2013). Thus, in addition to modifying the spatial location of bone marrow blood vessels (Prisby *et al.* 2011), PTH treatment modified the functional properties of the bone vasculature and enhanced the sensitivity of the vascular endothelium to agonists other than PTH (i.e., acetylcholine, ACh) (Prisby *et al.* 2013). Such endothelial cell modifications potentially enable bone blood vessels to enhance vasodilation in response to a myriad of locally released factors that mediate action through NO signaling and deliver more blood flow to bone. Further, this investigation demonstrated a relation between PTH-induced trabecular bone accrual and peak endothelium-dependent vasodilation, illustrating that PTH may influence bone mass via NO signaling in vascular endothelial cells (Prisby *et al.* 2013).

Enhanced blood flow following intermittent PTH administration has been documented. For example, post-menopausal women with osteoporosis experienced increased uptake of radiopharmaceutical technetium-99m methylene diphosphonate (^{99m}Tc -MDP) in the whole skeleton and to the calvarium, mandible, spine and upper and lower extremities following 18 months of teriparatide treatment (Moore *et al.* 2010). The presence of ^{99m}Tc -MDP

in the skeleton serves as a clinical indicator of bone blood flow (Blake *et al.* 2001). In a mouse model, trabecular bone volume in the distal femoral metaphysis and L5 vertebra and hind limb tissue perfusion were not altered following 4 weeks of intermittent PTH 1–34 administration (Gohin *et al.* 2016). Caution must be used in the interpretation of these data since the hind limb tissue perfusion measurements were not limited to the skeleton. Acute rises in bone perfusion (i.e., within 5–15 min) were observed in the whole hind limb as measured by laser Doppler imaging and in the cortical diaphysis as assessed by the augmented presence of procion red fluorescence following an injection of PTH (Gohin *et al.* 2016). Unfortunately, this measure was not obtained following the 4-week protocol when cortical bone volume and bone formation rate were enhanced (Gohin *et al.* 2016). The PTH-induced rise in hind limb tissue perfusion and enhanced cortical bone volume were blocked and blunted, respectively, with NO inhibition, highlighting the importance of this signaling cascade (Gohin *et al.* 2016). These data coincide with previous descriptions of the reliance on NO signaling for bone blood vessels (Prisby *et al.* 2013), bone blood flow (Kapitola & Zák 2003) and bone (Prisby *et al.* 2013) with PTH treatment. Fourteen days of intermittent administration of PTH 1–84 in mice augmented blood vessel number (+16% and +34%, respectively) and mean vessel volume (+70% and +45%, respectively) in the tibial metaphysis and diaphysis and increased tibial perfusion by 27% vs controls (Roche *et al.* 2014).

Interestingly, the two mouse studies highlight the independent influence of PTH on bone blood vessels and bone. Alterations in bone perfusion and bone vascular parameters were observed at time points when bone parameters were unaltered (Roche *et al.* 2014, Gohin *et al.* 2016). However, rapid bone cellular activities following an injection of PTH have been observed. Fifteen minutes following PTH (50 $\mu\text{g}/\text{kg}$) administration to 7-day-old postnatal rats, mast cells on bone surfaces underwent degranulation and by 1-h osteoclast numbers had risen (Nakamura *et al.* 1996). Additionally, the ruffled border and clear zone of the osteoclasts were augmented at 30 min and 30–90 min, respectively, in thyroparathyroidectomized rats following administration of 50U of purified bovine PTH (Holtrop *et al.* 1979). Osteoclast cell size was augmented by 90 min (Holtrop *et al.* 1979). In contrast, rats with an intact thyroid and parathyroid glands demonstrated no change in osteoclast size or ruffled border area with PTH but the clear zone was augmented at minute 30 (Holtrop *et al.* 1979). Six hours following injection of parathyroid extract, osteoblasts demonstrated

numerous dense bodies and swollen mitochondria (Cameron *et al.* 1967). However, uncertainty exists as to whether these changes translate into physiological activity or were a result of toxicity from the large dose parathyroid extract (4 units per gram) (Cameron *et al.* 1967). Data regarding the kinetic activities of osteoblasts during earlier time points following PTH injection are lacking. Regardless, augmented bone perfusion is observed rapidly (i.e., within 5–15 min) following acute injections of PTH (Kapitola & Zák 2003, Roche *et al.* 2014, Gohin *et al.* 2016) as opposed to the 15-min-to-6h delay for mast cells, osteoclasts and osteoblasts (Cameron *et al.* 1967, Holtrop *et al.* 1979, Nakamura *et al.* 1996), leaving one to speculate the involvement of bone blood vessels in the paracrine activation of bone cells.

Overall, PTH is a powerful stimulator of the bone vascular network in which it alters the spatial location of bone marrow blood vessels, enhances the vasodilator capacity of bone blood vessels, may elicit bone angiogenesis and augments bone blood flow, either after acute or chronic administration. It would be beneficial to ascertain whether intermittent PTH administration serves to deliver blood flow to more regions of bone tissue as reported following long-term (18 months) treatment in humans (Moore *et al.* 2010) and subsequent to chronic (10–12 weeks) exercise training in rats (Stabley *et al.* 2014). Additionally, data indicate independent actions of PTH on bone blood vessels and bone, whereby the effects may occur more rapidly in the vasculature. Finally, NO is an important regulator utilized by PTH to exert its actions on both blood vessels and bone.

Estrogen

Estrogen regulates bone metabolism in females and males and bone accrual during puberty is driven by 17 β -estradiol (Khalid & Krum 2016). The maintenance of bone mineral density is also dependent upon estrogen, particularly 17 β -estradiol, that interacts with the estrogen receptors α and β (Khalid & Krum 2016). Several mechanisms by which estrogen serves to build or protect bone mass have been suggested. For example, estrogen induced osteoclast apoptosis (Kameda *et al.* 1997, Kousteni *et al.* 2002). Additionally, the effects of estrogen on bone formation includes increased protein content for the estrogen receptors α and β , osteoprotegerin (i.e., the decoy receptor for receptor activator of nuclear factor K-B ligand (RANKL)) and RANKL (Bord *et al.* 2003). Estrogen also inhibits the

apoptosis of osteoblasts and osteocytes (Kousteni *et al.* 2001). Estrogen deficiency is linked with low bone mass (Nilas & Christiansen 1987), as plasma levels of estrogen correspond with low bone mass or bone loss (Johnston *et al.* 1985, Wronski *et al.* 1989). Following menopause, the rapid phase of bone loss occurs within 5–10 years; however, bone loss and fracture are mitigated by estrogen replacement (Ettinger *et al.* 1985).

Estrogen receptors are present on endothelial cells (Brandi *et al.* 1993) and, therefore, the influence of estrogen deficiency on bone blood vessels must be considered in regards to bone metabolism. The role of the vascular system in relation to bone homeostasis, as dictated by estrogen, can be deciphered by examining the effects of estrogen loss and replacement. Estrogen deficiency influences the vasomotor activity of bone blood vessels and/or alters their quantity in bone. Negative alterations in one or both will ultimately affect the delivery of blood to the skeleton. Bone loss with estrogen deficiency has been examined by several investigators who sought to determine the associated bone vascular consequences; however, the data have provided mixed results. In some investigations, estrogen deficiency via ovariectomy (OVX) served to (1) diminished trabecular bone volume and area, bone mineral density, bone biomechanical properties, erythropoietic marrow area, vascular density, vascular volume, immunohistochemical expression of vascular endothelial cell growth factor (VEGF) in bone, and bone perfusion and (2) increased the area of sinusoidal capillaries and marrow fat cell area (Laroche *et al.* 1996, Griffith *et al.* 2010, Ding *et al.* 2011). The area of sinusoids in the marrow coincided with bone resorption ($r=+0.46$), trabecular bone volume ($r=-0.54$) and trabecular number ($r=-0.60$) (Laroche *et al.* 1996). Interestingly, 15 days following OVX in mice, trabecular bone volume was not diminished but tibial bone perfusion and vascular density and volume in the marrow were reduced (Roche *et al.* 2014). Treatment with bisphosphonates prevented bone loss (Laroche *et al.* 1996) and attenuated the declines in bone perfusion (Roche *et al.* 2014) with OVX, but did not alter bone vascular density, volume and blood vessel size (Laroche *et al.* 1996, Roche *et al.* 2014). In contrast, OVX and orchidectomy (i.e., castration) in rats demonstrated increased bone blood flow (Kapitola & Kubícková 1990, Egrise *et al.* 1992, Kapitola *et al.* 1994) with enhanced bone turnover and bone loss (Kapitola & Kubícková 1990, Egrise *et al.* 1992, Kapitola *et al.* 1994) and diminished bone density and ash weight (Kapitola & Kubícková 1990, Kapitola *et al.* 1994) in young rats.

Sequentially, the physiological events following OVX demonstrate that alterations in bone vascular parameters occur more rapidly than bone parameters. For example, mRNA expression of VEGF 120 and 164 (−15% and −19%, respectively), endothelial and inducible NO synthase (−38% and −34%, respectively), and fibroblast growth factor-2 (−18%) in the proximal tibial metaphysis were reduced 3 days following OVX (Mekraldi *et al.* 2003). Declines in bone blood vessel number occurred at 7 days and reduced trabecular bone volume did not occur until day 14 (Mekraldi *et al.* 2003). Vascular endothelial growth factor and fibroblast growth factor-2 are angiogenic factors and NO synthase is responsible for the production of NO. Similarly, vascular density and volume were reduced by ~13–15%, bone vascular resistance augmented by ~36% and tibial perfusion reduced by 28% vs sham-operated controls following 15 days of OVX in C57BL/6 mice; i.e., at time points when trabecular bone volume and cortical thickness in the tibia were unaltered (Roche *et al.* 2013, 2014). Collectively, these data suggest that estrogen deficiency influences the bone vascular system prior to impacting bone.

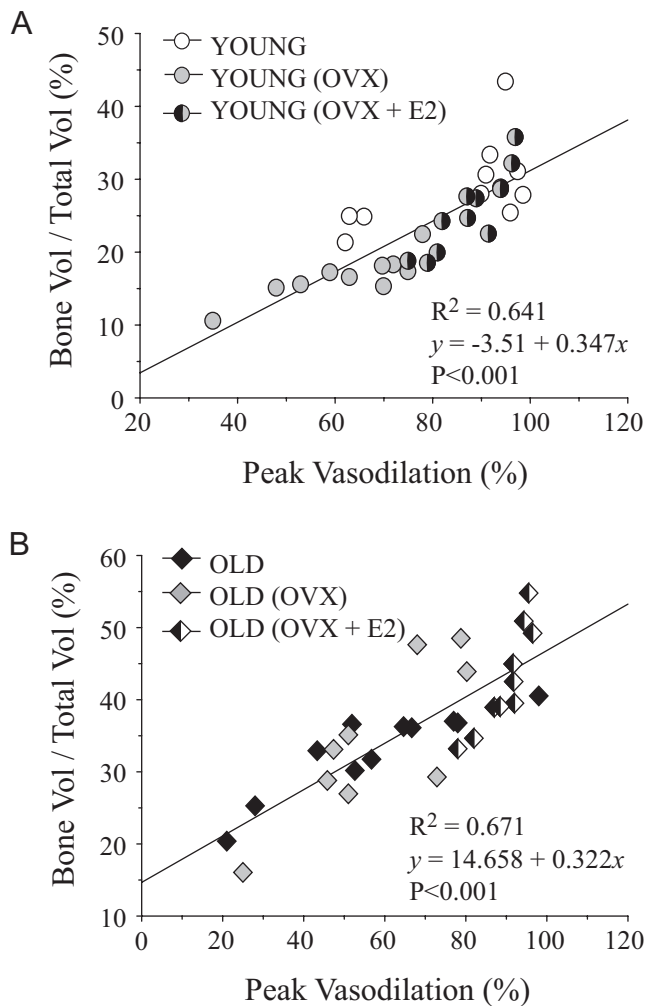
Data regarding enhanced vasoconstrictor and diminished vasodilator activity of bone blood vessels support the results in rats and mice demonstrating declines in bone perfusion with estrogen loss (Griffith *et al.* 2010, Roche *et al.* 2014). Six weeks after bilateral OVX in rabbits, force development in femoral metaphyseal arteries was significantly enhanced following stimulation with norepinephrine and endothelin-1, suggesting increased vasoconstrictor responsiveness of these vessels. However, no changes in force development were observed in diaphyseal arteries of the femur (Hansen *et al.* 2001). Additionally, OVX did not alter vasodilator responsiveness to ACh or substance P in metaphyseal and diaphyseal arteries (Hansen *et al.* 2001), indicating that vasodilator mechanisms are unaltered. Unfortunately, no bone parameters were reported.

In addition, OVX in young (4–6 months) rats reduced ACh-mediated, endothelium-dependent vasodilation of the femoral PNA and reduced femoral trabecular bone volume (Prisby *et al.* 2012). Endothelium-dependent vasodilator capacity (Prisby *et al.* 2012) and blood flow responses (Kapitola *et al.* 1997) following OVX were related to the ability of endothelial cells to regulate vasodilation via NO-mediated signaling. Similar to the bone data in OVX mice (Roche *et al.* 2013, 2014), trabecular bone volume and endothelium-dependent vasodilation in old (22–24 months) rats were not altered by OVX (Prisby *et al.* 2012). Strong relations were established, however, between peak endothelium-dependent vasodilation of the femoral

PNA and trabecular bone volume in both young and old animals (Prisby *et al.* 2012). In other words, rats with high vasodilator capacity also demonstrated high trabecular bone volume in the distal femur (Fig. 2). Interestingly, animals with low circulating estrogen (i.e., young OVX, old intact and old OVX) displayed poor associations between trabecular bone volume and plasma estrogen levels and uterine mass, highlighting that circulating estrogen has a minimal effect on trabecular bone mass in these groups (Figs 3 and 4A and B). However, strong relations were observed between peak endothelium-dependent vasodilation and trabecular bone volume in these low estrogen animals (Prisby *et al.* 2012). Thus, bone vascular function, as mediated by endothelial NO signaling, is coupled more to bone volume than estrogen status (Figs 3 and 4C).

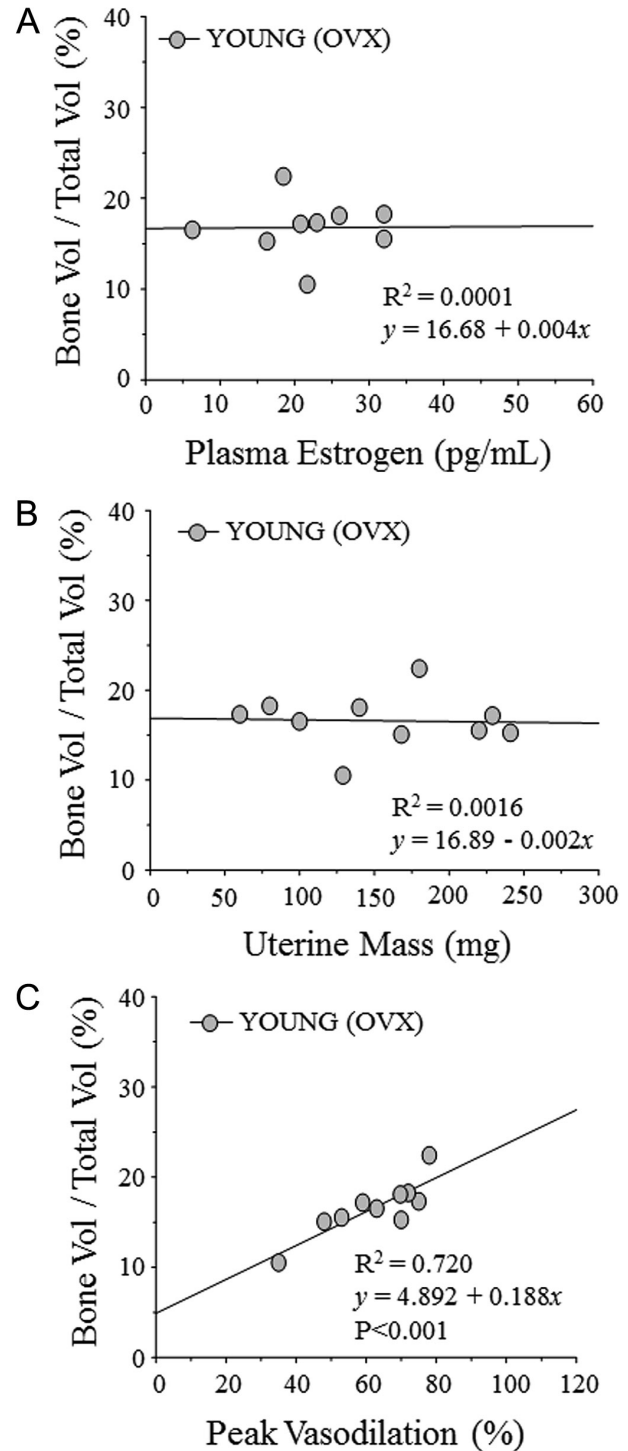
The importance of NO-mediated vasodilation with the loss of estrogen is also highlighted in an investigation examining vasomotor responsiveness in large conduit vessels (i.e., aorta and femoral artery). Following 8 weeks of OVX, ACh-mediated, endothelium-dependent relaxation was impaired and phenylephrine-induced contraction was augmented in OVX vs control rats (Griffith *et al.* 2010). Thus, large conduit vessels external to bone also demonstrate reduced relaxation and enhanced contraction with estrogen deficiency, illustrating the global effects of this hormone. The differences in responsiveness to phenylephrine between OVX and control rats disappeared in the presence of the NO synthase inhibitor, L-NAME and no differences were observed between the groups in endothelium-independent relaxation to the NO donor, sodium nitroprusside (Griffith *et al.* 2010). Even though conduit vessels (i.e., aorta and femoral artery) were examined in this investigation, results correspond with bone blood vessel data demonstrating a reliance on NO-mediated, endothelium-dependent vasodilation and no change in vascular smooth muscle cell responsiveness with OVX (Prisby *et al.* 2012). Further, these data demonstrate that the effects of estrogen deficiency are systemic and not localized to bone blood vessels. Thus, bone loss with estrogen deficiency may result directly from bone cellular dysfunction or from dysfunction within the vascular system.

Estrogen replacement with OVX reverses the changes in bone and vascular parameters. For example, estrogen repletion with OVX maintained (young rats) and augmented (old rats) endothelium-dependent vasodilation and trabecular bone volume (Prisby *et al.* 2012). Correspondingly, administration of 17 β -estradiol to OVX animals restored the observed declines in

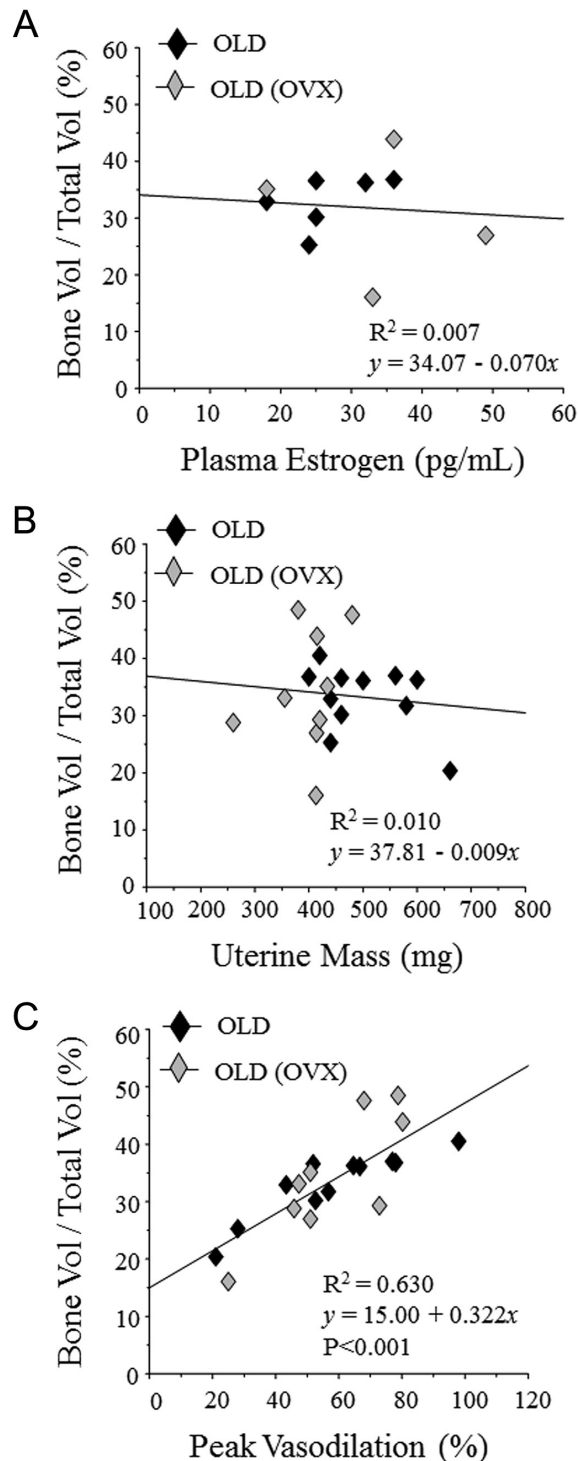
**Figure 2**

Regression analyses between peak endothelium-dependent vasodilation (%) of the rat femoral PNA and trabecular bone volume (%) of the distal femur in (A) young and (B) old ovariectomized (OVX) and estrogen-replaced (E2) Fischer-344 rats. The regression analyses were $P < 0.001$ and $P < 0.001$ in (A) young and (B) old rats, respectively. Values are means \pm s.e. Adapted, under the terms of the Creative Commons Attribution License, from Prisby *et al.* (2012).

mRNA expression of VEGF 120 and 164, bone blood vessel number and bone volume in the proximal tibial metaphysis (Mekraldi *et al.* 2003). When given in supraphysiological doses, massive bone formation occurred in the epiphysis, metaphysis and diaphysis of tibiae and femora from immature female and male mice; yet, the vascular pattern within the bone was unaltered (Brookes & Lloyd 1961). These data suggest that estrogen repletion enhances the vasodilator capacity of bone blood vessels. However, administration of estradiol benzoate in intact and OVX rats diminished blood flow to the tibia and enhanced bone density and ash weight in the OVX group only (Kapitola & Kubicková 1990).

**Figure 3**

Scattergrams illustrating the relation between (A) plasma estrogen, (B) uterine mass and (C) peak endothelium-dependent vasodilation of the rat femoral PNA. Bone volume (BV/TV, %) was not correlated to (A) plasma estrogen nor (B) uterine mass in young Fischer-344 rats with low estrogen status (i.e., ovariectomized (OVX)). However, BV/TV and (C) peak vasodilation of the femoral PNA were significantly ($P < 0.001$) and positively related. Adapted, under the terms of the Creative Commons Attribution License, from Prisby *et al.* (2012).

**Figure 4**

Scattergrams illustrating the relation between (A) plasma estrogen, (B) uterine mass and (C) peak endothelium-dependent vasodilation of the rat femoral PNA. Bone volume (BV/TV, %) was not correlated to (A) plasma estrogen nor (B) uterine mass in old Fischer-344 rats with low estrogen status (i.e., ovariectomized (OVX) and old age). However, BV/TV and (C) peak vasodilation of the femoral PNA were significantly ($P < 0.001$) and positively related. Adapted, under the terms of the Creative Commons Attribution License, from [Prisby et al. \(2012\)](#).

Given the totality of data with estrogen deficiency and repletion, a clear picture does not emerge in regards to the direction of influence estrogen has on bone blood flow and bone vascular responses. However, the data seem to indicate that bone vascular changes occur at a rapid rate as opposed to the delayed responses in bone.

Vitamin D

Normal development and the maintenance of the skeleton is reliant upon vitamin D ($25(\text{OH})\text{D}_3$), such that the active form, $1\alpha,25(\text{OH})_2\text{D}_3$, is involved in calcium regulation and bone homeostasis via endocrine and autocrine functions ([Siddiqui & Partridge 2016](#)). In addition to vitamin D levels being associated with bone mineral density ([Bischoff-Ferrari et al. 2004](#)), $1\alpha,25(\text{OH})_2\text{D}_3$ is a treatment for post-menopausal osteoporosis ([Dong et al. 2013](#)). For example, calcitriol, a compound of vitamin D, augmented trabecular bone mass and cortical thickness in sham-operated and OVX rats ([Faugere 1986](#)).

Among other functions, vitamin D is theorized to regulate vascular tone ([Dalan et al. 2014](#)) and vitamin D receptors (VDRs) are present on endothelial and smooth muscle cells ([Andrukhova et al. 2014](#)). Knockout of the VDR receptor in young mice reduced NO bioavailability and NO synthase 3 gene expression and protein content ([Andrukhova et al. 2014](#)). The enzyme ($25(\text{OH})\text{D}_3$ - 1α -hydroxylase) that converts circulating, inactive $25(\text{OH})\text{D}_3$ to active $1\alpha,25(\text{OH})_2\text{D}_3$ is present in human renal endothelial cells, post-capillary venules from lymphoid tissue and in human umbilical vein endothelial cells ([Zehnder et al. 2002](#)). Further, $1\alpha,25(\text{OH})_2\text{D}_3$ transcriptionally regulates endothelial NO synthase ([Andrukhova et al. 2014](#)), providing evidence for an autocrine effect on vasodilation.

In humans, brachial flow-mediated dilation (i.e., a measure of NO-related endothelial cell function) was diminished in vitamin D-deficient, type 2 diabetic patients ([Yiu et al. 2011](#)). However, supplementation with vitamin D to improve endothelial cell function has produced equivocal results. Investigations in humans and animals have reported improved ([Harris et al. 2011](#), [Dong et al. 2013](#)) or no change in ([Gepner et al. 2012](#), [Yiu et al. 2013](#)) endothelium-dependent vasodilation with vitamin D supplementation. Given the dual role of vitamin D on the vascular and skeletal systems, it is plausible that this hormone would influence bone blood vessels and regulate bone blood flow. To date, examination of acute or chronic effects of vitamin D on bone vascular function has not been conducted.

Calcitonin and calcitonin gene-related peptide

Calcitonin is a hormone produced in the thyroid gland and, due to its anti-resorptive actions, is FDA approved for the treatment of osteoporosis and diseases with high bone turnover (Naot & Cornish 2008, Khosla 2011). Calcitonin gene-related peptide (CGrP) is produced as a result of alternative splicing of the calcitonin gene at the tissue level (Siddiqui & Partridge 2016) and is released from nerve terminals (Hill & Elde 1988). Both peptides bind to the CT receptor (Inzerillo *et al.* 2002). While not observed on osteoblasts (Naot & Cornish 2008), the CT receptor is present on osteoclasts (Ikegame *et al.* 1995) and perhaps osteocytes (Gooi *et al.* 2010). Calcitonin was shown to inhibit apoptosis of osteoblasts and osteocytes (Plotkin *et al.* 1999), demonstrating a potential indirect influence on bone formation. Calcitonin gene-related peptide activates CT receptors on osteoclasts (Zaidi *et al.* 1987) to reduce their activity and diminish bone resorption (Zaidi *et al.* 1987). The net effect enhances osteogenesis, mineralization and calcium uptake into bone (Bernard & Shih 1990, Ancill *et al.* 1991). However, mice deficient in α CGrP or calcitonin demonstrated low and high bone masses, respectively (Schinke *et al.* 2004) and overexpression of osteoblastic CGrP in mice augmented trabecular bone density and volume in the femoral metaphysis (Ballica *et al.* 1999). Calcitonin gene-related peptide is localized in nerve fibers (Hill & Elde 1988) observed between trabeculae in the epiphysis and at the epiphyseal plate (Shih & Bernard 1997). These nerve fibers are also located in Haversian and Volkmann canals (Shih & Bernard 1997); i.e., the channels for blood vessels in the cortical diaphysis. Thus, CGrP is uniquely positioned to regulate vasomotor activity.

Bone resistance arteries are responsive to the same factors that induce bone accrual and remodeling. For example, data confirm that CGrP elicits vasodilation in rat femoral PNAs, whereby modest vasodilation (33%) occurred to increasing concentrations (RD Prisby, unpublished observations). Similarly, relaxation to CGrP was observed in pre-constricted arterial segments from cancellous bone of pigs (Lundgaard *et al.* 1997). However, not all species are responsive, such that bone vascular resistance in rabbits was unaltered by CGrP (Vendégh *et al.* 2010). Further, tibial (–33%) and femoral (–34%) marrow flow velocities and systemic blood pressure (–26%) were reduced following administration of CGrP (Vendégh *et al.* 2010). The authors concluded that since marrow vascular resistance remained unchanged, the declines in

bone perfusion may have resulted from the shunting of blood from bone to other tissue beds (e.g., muscle, skin and fat) undergoing active vasodilation (Vendégh *et al.* 2010). Given the alterations in bone cellular activity in the presence of CGrP (Hohmann *et al.* 1983, Zaidi *et al.* 1987, Bernard & Shih 1990, Zheng *et al.* 2016), its ability to induce vasodilation (RD Prisby, unpublished observations; Lundgaard *et al.* 1997) at the level of the resistance vessel is suggestive of a role in regulating bone blood flow.

In regards to calcitonin, the vasoconstrictor and vasodilator influences on the bone vasculature is complex. For example, calcitonin elicited dose-dependent increases in perfusion pressure, perhaps via direct actions on vascular smooth muscle cells to induce vasoconstriction (Driessens & Vanhoutte 1981) and calcitonin administered for 7 days and 7 weeks diminished skeletal blood flow in patients with Paget's disease (Wootton *et al.* 1978). In contrast, calcitonin administered for 30 days had no effect in reducing the elevated tibial and femoral bone blood flow induced by paraplegia in rats; however, it did augment femoral blood flow in intact control animals (Schoutens *et al.* 1988). Correspondingly, the paraplegic rats treated for 30 days with calcitonin demonstrated augmented tibial bone volume, periosteal apposition rate and calcium accretion in comparison to paraplegic rats with no treatment (Schoutens *et al.* 1988).

Taken together, the vasomotor effects of calcitonin on the bone vascular system is difficult at this point to ascertain. The vasoconstrictor influence of calcitonin on the bone vascular system (Wootton *et al.* 1978, Driessens & Vanhoutte 1981) appears divergent from its role as a treatment for osteoporosis. Further, CGrP released from nerve terminals or produced locally may provide vasodilator cues and stimulate bone formation. The data are divergent, however, and additional work is warranted.

Metabolic byproducts

Mechanical and hormonal factors stimulate the release of VEGF from osteoblasts to ultimately augment blood flow and the delivery of O₂, nutrients and precursor cells for bone formation. For example, osteoblast-derived VEGF-initiated repair of small bone defects via angiogenesis and inflammation (Hu & Olsen 2016) and, along with Hif-1 α and Hif-2 α , VEGF was reduced in the distal femur of OVX mice (Zhao *et al.* 2012). Further, VEGF was augmented at the tibial growth plate in juvenile mice with administration of 1 α ,25(OH)₂D₃ (Lin *et al.* 2002) and its variants were

produced by low and high mechanical deformations in human osteoblasts (Faure *et al.* 2008). Thus, under the control of bone anabolic agents, bone cells are capable of enhancing blood flow via angiogenesis. What should also be considered is the regulation of bone blood flow, not via angiogenesis, but by enhanced vasodilation or reduced vasoconstriction of nearby blood vessels as a result of bone cellular metabolism.

Metabolic vasodilation is necessary for delivering blood to tissues such as skeletal muscle and the heart (Rowell 1993, Barvitenko *et al.* 2013), particularly in the face of increased metabolism. In reference to dynamically active skeletal muscle, many factors released into the interstitial space produce vasomotor activity of the nearby vasculature. These factors (e.g., hydrogen ions, carbon dioxide, adenosine nucleotides, potassium, phosphate ions, lactate, intermediates of the tricarboxylic acid cycle, vasoactive peptides, etc.), in addition to changes in the partial pressure of oxygen (pO_2), directly and indirectly stimulate vasodilation (Rowell 1993). They represent the various means by which metabolism serves to augment tissue perfusion. The effects produced on the vasculature by these factors can occur independently; however, collectively they have a far stronger influence (Rowell 1993).

Similar to skeletal muscle, which increases its metabolic activity through movement, the metabolic activity of bone cells is augmented during cellular differentiation, and during processes such as bone resorption and formation. Bone cells produce many of the metabolic byproducts observed in active skeletal muscle (e.g., carbon dioxide, lactate, hydrogen ions, phosphate ions, adenosine diphosphate (ADP) and citric acid) (Borle *et al.* 1960a, Karner & Long 2016). For example, differentiating osteoblasts rely heavily upon glucose utilization (Esen *et al.* 2013, 2015) and produce lactate as a result (Felix *et al.* 1978). During osteoclastogenesis, mitochondrial mass within the cell is high and the formation of osteoclasts correlates to elevated rates of oxidative phosphorylation, as reviewed by Lemma and coworkers (2016). Yet, during bone resorption, glycolysis is the predominant pathway (Lemma *et al.* 2016). In addition, bone cells have the capacity to utilize amino and fatty acids (Karner & Long 2016). Fatty acids undergo β -oxidation when they enter the cell, producing acetyl-coA for the tricarboxylic acid cycle (Karner & Long 2016). Since metabolic activity is continual in bone and bone marrow cells, we must underscore how these factors released during cellular differentiation and bone formation and resorption contribute to the local regulation of bone blood flow.

The regulation of metabolic activity during bone formation and resorption is heavily dependent upon the pO_2 at the bone surface and the production of acids, primarily lactate, that allows for continued bone degradation (Borle *et al.* 1960b). Bone marrow pO_2 is $\sim 7\%$ in normal, healthy individuals (Harrison *et al.* 2002). Altering pO_2 to mimic physiological and pathophysiological conditions has revealed cell responsiveness to these changes. For example, reducing pO_2 to 2% in a 7-day mouse marrow culture augmented the number (+3.2-fold) and activity (+21-fold) of osteoclasts (Arnett *et al.* 2003). Additionally, bone nodule formation by osteoblasts was reduced (-10 -fold) and almost completely inhibited as a result of moderate (1–2% O_2) and severe (0.2% O_2) hypoxia, respectively (Utting *et al.* 2006). Reduced pO_2 is a powerful stimulator for vasodilation in other vascular beds (Spilk *et al.* 2013) and no doubt produces similar responses in the bone vascular system. In order to rapidly restore bone pO_2 , vasodilation of bone blood vessels would be the foremost response. Reduced pO_2 is usually accompanied by an acidic environment in bone. The production of acids, lactate in particular, during enhanced metabolism serves to lower bone pH. *In vitro*, resorption pit area on cortical bone slices was increased (+14-fold) with a 0.6 decline in pH of the cell culture medium (Arnett & Dempster 1986) and reducing pH from 7.08 to 6.82 increased resorption of dentine disks by osteoclasts (Hoebertz *et al.* 2001). Reduced pO_2 and pH in bone enhances metabolic activity in osteoclasts and, with augmented metabolic activity, a rise in bone blood flow is requisite.

Systemic alterations in blood gases and pH were demonstrated to regulate bone blood flow. For example, when hypercapnia (i.e., carbon dioxide retention; 65 ± 1 mmHg) and acidosis (7.14 ± 0.02 units) were introduced to anesthetized canines, blood flow to the sternum, rib and femoral marrow were augmented 166, 67 and 233%, respectively (Gross *et al.* 1979). Further, reduced pH or increased partial pressure of carbon dioxide in bone (Brookes 1966, Brookes & Revell 1998) was theorized as the stimuli for bone accrual following long-term femoral vein ligation (Brookes 1966, Brookes & Singh 1972). Additional metabolic byproducts elicit changes in bone cell metabolism and vascular responses. For example, extracellular ADP and ATP produced significant increases in pit formation with acidified cell culture medium and induced the formation of osteoclasts on dentine and ivory disks; however, adenosine and adenosine monophosphate (AMP) had

no effect (Morrison *et al.* 1998, Hoebertz *et al.* 2001). Introduction of adenosine into the brachial vein of conscious dogs reduced vascular resistance and augmented blood flow to the sternum by ~37%; however, the adenosine also reduced arterial pressure by 29% (Gross *et al.* 1979).

While not all metabolic byproducts have been examined, data illustrate how alterations in their concentrations stimulate osteoclasts to resorb bone and induce vasodilation of the local vasculature to augment blood flow. As mentioned previously, enhanced bone blood flow augments bone interstitial fluid flow and pressure to stimulate the release of paracrine and autocrine factors from bone cells. Additionally, augmented shear stress on vascular endothelial cells elicit release of paracrine cues. Thus, we can begin to appreciate the integrative nature of the vascular and skeletal systems, whereby augmented metabolism in the form of catabolic activity may quickly lead to bone anabolism by participation of the blood vessel at the core of bone basic multicellular unit or bone remodeling compartment (Fig. 5). The close proximity of

blood vessels to osteoclasts and osteoblasts allows for this intimate bi-communication.

The vascular endothelium

As presented in the previous section, early studies speculated that bone modulates the blood flow that it receives via the production of metabolic byproducts (Davis 1992). We now know bone cells release a myriad of factors that modulate blood vessel function (Alam *et al.* 1992, Delany & Canalis 1997, Ishida *et al.* 2002, Brandi & Collin-Osdoby 2006, Li *et al.* 2016). What is not greatly appreciated is the reciprocal relation whereby bone blood vessels release factors (e.g., insulin-like growth factor-1, endothelin-1, prostacyclin, RANKL, interleukin-11, PGE₂, NO, ATP, ADP, AMP, adenosine, etc.) that stimulate bone cellular activity (Alam *et al.* 1992, Fiorelli *et al.* 1994, Shinozuka *et al.* 1994, Zhang *et al.* 1998, Kage *et al.* 1999, Ishida *et al.* 2002, Brandi & Collin-Osdoby 2006, Gonzalez *et al.* 2015, Wen *et al.* 2015, Gutterman *et al.* 2016).

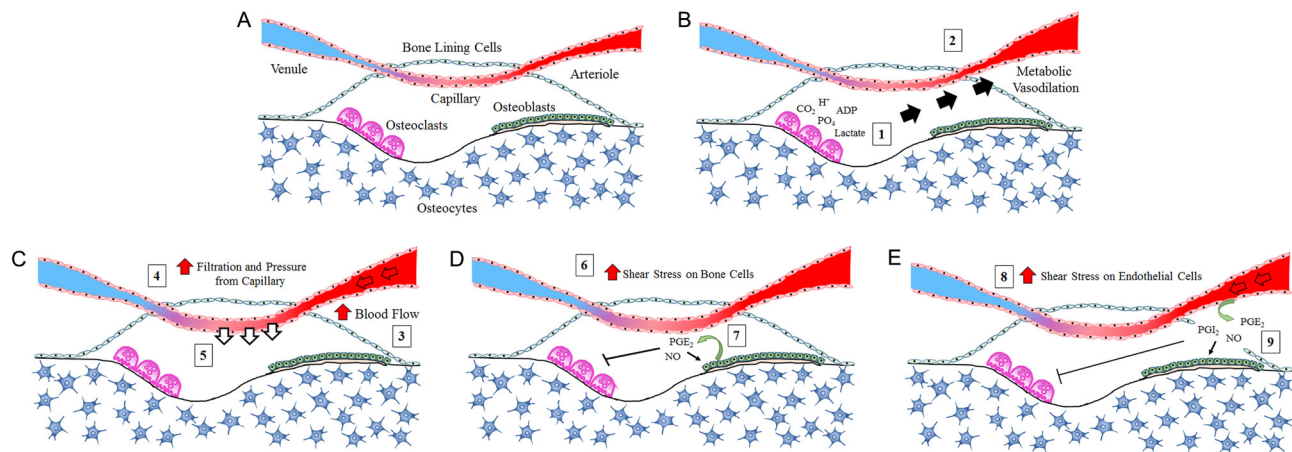


Figure 5

A sequential scenario by which metabolic activity leads to enhanced vasodilation of the surrounding arterioles and arteries. The schematic represents the bone remodeling compartment with trabecular bone containing osteocytes and the neighboring marrow occupied by osteoclasts, osteoblasts, bone lining cells and the vascular network (i.e., arteriole, capillary and venule). Panel A serves to orient the reader to the various cells and structures within this schematic. Vascular smooth muscle cells were omitted from the arteriole and venule for simplicity, thus, the cells being depicted are vascular endothelial cells. Panel B depicts osteoclasts actively resorbing bone (1). During the course of enhanced metabolism, the osteoclasts release factors (e.g., carbon dioxide (CO₂), hydrogen ion (H⁺), phosphate (PO₄), ADP, lactate, etc.) that diffuse to the arteriole and initiate metabolic vasodilation (2). The metabolic vasodilation of the arteriole causes ascending vasodilation of the feed and conduit arteries upstream (not depicted). This process is called Conducted Vasodilation and ensures a rise in blood flow to the surrounding tissue undergoing metabolism. Panel C illustrates how vasodilation and the subsequent rise in blood flow (3) augments filtration and pressure from the capillary (4) into the bone interstitial space (5). The increased bone interstitial pressure and fluid flow generate shear stress on bone cells (Panel D, 6). Shear-mediated release of PGE₂ and NO from osteoblasts (7) serves to enhanced osteoblast activity and reduced osteoclast activity, slowing down bone degradation and ramping up bone formation. In addition, enhanced blood flow as a result of conducted vasodilation augments shear stress on vascular endothelial cells (Panel E, 8). As a result, vascular endothelial cells release factors (e.g., NO, PGE₂, prostacyclin (PGI₂)) that diffuse into the bone interstitial space to stimulate osteoblast activity and inhibit osteoclast activity (9). The theories of vascular contribution to enhanced bone formation were previously put forth by the laboratory of Michael Delp (Colleran *et al.* 2000). Note that these processes do not have to begin with osteoclast activity. For example, circulating factors that induce vasodilation in the bone vascular network will serve to commence similar processes.

In order to meet the metabolic needs of various tissues with an adequate supply of blood, the arterial microcirculation regulates vascular resistance to control tissue blood flow, as reviewed by [Gutterman *et al.* \(2016\)](#). Only ~7% of body volume is occupied by the microcirculation; however, it is precisely distributed to provide nutrient delivery and remove waste products from every cell ([Gutterman *et al.* 2016](#)). Optimal microcirculatory function maintains tissue health because it provides blood flow, is the largest paracrine producer in the body, and as a result, modulates the function of the surrounding parenchyma ([Gutterman *et al.* 2016](#)). When factors are released from the endothelium, they travel in an abluminal direction to vascular smooth muscle cells to elicit a physiological response (e.g., hypertrophy, fibrosis or vasomotor activity) ([Gutterman *et al.* 2016](#)). Additionally, these factors pass beyond the vascular smooth muscle

cell layer in arteries and easily escape the endothelial cell layer of capillaries, eventually infiltrating the surrounding tissue ([Gutterman *et al.* 2016](#)). Thus, vascular endothelial and smooth muscle cells provide paracrine cues to the underlying parenchyma ([Gutterman *et al.* 2016](#)).

Endothelial cells have the ability to release ATP ([Vizi *et al.* 1992](#), [Shinozuka *et al.* 1994](#)). As discussed in the previous section, ATP and ADP influence osteoclast activity ([Morrison *et al.* 1998](#), [Hoebertz *et al.* 2001](#)) and adenosine reduced vascular resistance and increased blood flow to the sternum ([Gross *et al.* 1979](#)). Cortical blood vessel- and osteocyte-derived endothelial NO synthase was augmented rapidly after fracture in rat tibiae and was assumed to contribute to early increases in blood flow to the fracture site ([Corbett *et al.* 1999](#)). Targeted disruption of the NO gene reduced bone mineral density and bone volume, cortical thickness, predicted biomechanical

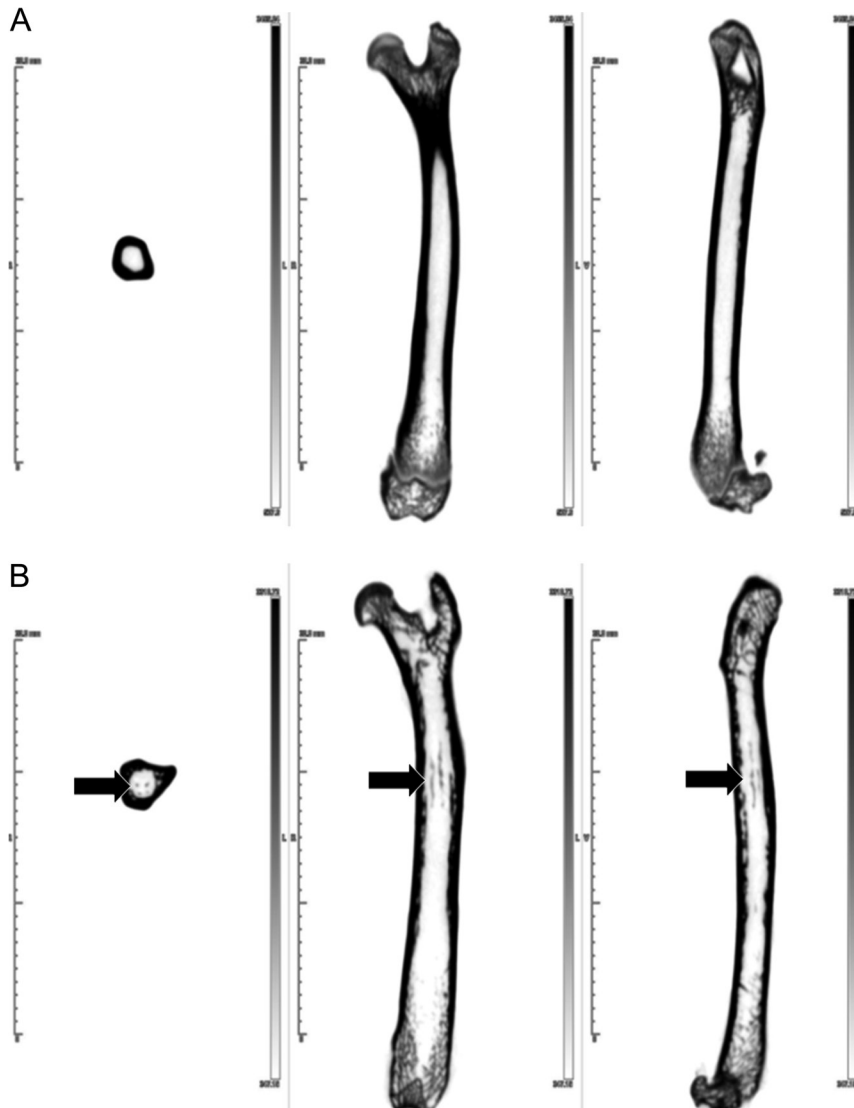


Figure 6

μ CT imaging of young and old rat femora. Panels represent cross-sections of the mid-diaphysis and frontal and sagittal views from (A) young (4–6 months) and (B) old (22–24 months) male, Fischer-344 rats, respectively. The arrows denote ossified blood vessels evident in the diaphyseal marrow of the old femur. Femora were imaged with non-contrast CT using a MicroCAT II CT scanner (Imtek Inc.; Siemens), yielding reconstructed voxels of $103 \times 103 \times 103 \mu\text{m}$. X-rays were generated at 80 kVp and 500 mA.

strength and bone formation rate in 6- to 9-week-old endothelial NO synthase knockout mice (Aguirre *et al.* 2001, Armour *et al.* 2001); however, these changes were corrected or not observed by 12 weeks (Aguirre *et al.* 2001, Sabanai *et al.* 2008). These data highlight the importance of NO in early bone development.

In addition to NO, vascular endothelial cells release endothelium-dependent hyperpolarizing factors, which cause vasodilation and enhances blood flow in numerous tissues (Barvitenko *et al.* 2013). Prostaglandin E₂ released from endothelial cells may serve to augment bone mass (Akamine *et al.* 1992) and insulin-like growth factors, also released from blood vessels, are important for bone growth and development (Baker *et al.* 1993). Several other chemokines, matrix molecules, cytokines and growth factors released by blood vessels may regulate a variety of bone cellular activities (e.g., recruitment, proliferation, differentiation, etc.) necessary for function and survival (Brandi & Collin-Osdoby 2006). How these various factors contribute to the optimal (and perhaps sub-optimal) functioning of bone and bone marrow cells is too vast for discussion here. However, the reader is directed to an excellent review on the topic (Brandi & Collin-Osdoby 2006). At any rate, the microcirculation could be considered a bone anabolic agent that modulates the functioning of bone cells as well as being a regulator of bone blood flow.

Dysfunction of the microcirculation has been suspected in a variety of disease states (Gutterman *et al.* 2016). As endothelial cells are intricately important for bone and bone marrow function, it is important to consider how pathology of the vascular system may impact these two other organs. Age- and disease-related vascular dysfunction occurs in bone (Prisby *et al.* 2007, Prisby *et al.* 2008, 2012, 2015, Stabley *et al.* 2015) as it does in other tissue beds (Lesniewski *et al.* 2008, 2011, Kang *et al.* 2009, Park *et al.* 2012). For example, a recently identified capillary subtype, found in mouse bone marrow and theorized to regulate angiogenesis, declines in advanced age (Kusumbe *et al.* 2014). Further, arteriosclerotic lesions were observed in arteries from human femora, with disease progression being about 10 years more advanced than what was observed in similar arteries outside of the skeleton (Ramseier 1962). Additionally, the progressive ossification of bone marrow blood vessels (i.e., the presumed conversion of blood vessels into bone; Fig. 6) with advancing age leads to 'microvascular dead space' (Prisby 2014). 'Microvascular dead space' has been defined as a loss of vasomotor

function (i.e., vasodilation and vasoconstriction) and patency in bone marrow blood vessels (Prisby 2014) and presumably has dramatic impacts on the delivery of bone blood flow, bone interstitial fluid flow and pressure and release of paracrine factors from the vascular endothelium. In addition, progressive ossification of bone marrow blood vessels most likely contributes to and precipitates the centripetal blood flow direction in long bones observed in advanced age, as opposed to the centrifugal direction observed in youth (Fig. 7; Prisby 2014). Thus, when considering age- and disease-related pathologies of bone, the independent pathologies of the vascular system must be considered. In other words, pathologies that originate, develop independent from the skeleton and cause dysfunction in the vascular system may proceed and contribute to developing osteopathology. Given the interconnectedness of bone blood vessels and bone, these physiological and pathological concepts may not be so easily separable.

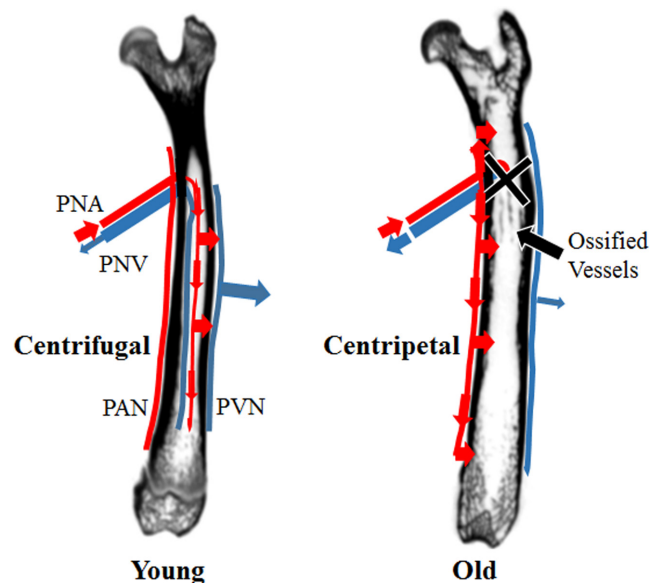


Figure 7

Schematic representing centrifugal (young) and centripetal (old) blood flow supply in long bones (Brookes & Revell 1998). Centrifugal blood flow indicates that blood enters the long bone through the principal nutrient artery (PNA), flows into the middle of the marrow space, radiates to the endosteal network and exits primarily via the periosteal venular network (PVN). Blood is also capable of exiting via the principal nutrient vein (PNV). Progressive ossification of bone marrow blood vessels may contribute to and precipitate the transition of the blood supply from centrifugal in youth to centripetal in old age. By reducing or eliminating the patency of the bone marrow vasculature (i.e., the black X), the blood supply must predominately enter the long bone via the periosteal arterial network (PAN). Centripetal blood supply represents an abnormal blood flow pattern (Brookes 1973) and has been previously represented as a schematic by Thompson & Towler (2012).

Table 2 Effects of osteogenic agents on bone blood vessels.

	Outcome
Mechanical loading	
Acute	<ul style="list-style-type: none"> ↑ MAP, ↑ bone VR (Gross <i>et al.</i> 1979). ↑ Blood flow to distal femur metaphysis and marrow (Dominguez <i>et al.</i> 2010). ⇔ MAP, ↑ blood flow to tibia and femur bone regions and marrow, ↑ exercise hyperemia to varying bone regions (Stabley <i>et al.</i> 2014).
Chronic	<ul style="list-style-type: none"> ↑ Blood vessel number and ⇔ blood vessel number with anti-VEGF antibody (Yao <i>et al.</i> 2004). ↑ Bone capillarity (Viboolvorakul <i>et al.</i> 2009). ↑ Endothelium-dependent vasodilation in PNA (Dominguez <i>et al.</i> 2010).
PTH and PTHrP	
Acute	<ul style="list-style-type: none"> ↓, then ↑ in blood flow to various hind limb bones (Boelkins <i>et al.</i> 1976). ⇔ Blood flow to isolated tibia (Driessens & Vanhoutte 1981). ↑ Blood flow to tibia and distal femur (Kapitola & Zák 2003). ↑ Vasodilation in PNA (Benson <i>et al.</i> 2016). ↑ Whole hind limb perfusion, ↑ perfusion in cortical diaphysis (Gohin <i>et al.</i> 2016).
Intermittent	<ul style="list-style-type: none"> ↑ Blood flow to whole skeleton, calvarium, mandible, spine, and upper and lower extremities (Moore <i>et al.</i> 2010). ↓ Blood vessel number, relocation of smallest bone marrow blood vessels (Prisby <i>et al.</i> 2011). ↑ Endothelium-dependent vasodilation in PNA (Prisby <i>et al.</i> 2013). ↑ Blood vessel number and mean vessel volume, ↑ tibial perfusion (Roche <i>et al.</i> 2014). ⇔ Whole hind limb tissue perfusion (Gohin <i>et al.</i> 2016).
Estrogen	
Supplementation	<ul style="list-style-type: none"> ⇔ Vascular pattern in tibia and femur (Brookes & Lloyd 1961). ↑ Tibia blood flow with OVX, ↓ tibia blood flow in Intact+E2 and OVX+E2 (Kapitola & Kubícková 1990). ↑ VEGF 120 and 164 in proximal tibia metaphysis, ↑ blood vessel number (Mekraldi <i>et al.</i> 2003). ⇔ and ↑ endothelium-dependent vasodilation in PNAs from young and old rats, respectively, ⇔ endothelium-independent vasodilation in PNA from young and old rats (Prisby <i>et al.</i> 2012).
Estrogen loss	<ul style="list-style-type: none"> ↑ Femur blood flow at days 28–84 OVX (Egrise <i>et al.</i> 1992). ↑ Tibia and distal femur blood flow with OVX (Kapitola <i>et al.</i> 1994). ⇔ Femur blood flow, ↑ tibia and distal femur blood flow with OVX, ↓ tibia and distal femur blood flow in OVX and sham-operated rats with NO blocker (Kapitola <i>et al.</i> 1997). ↑ Area of sinusoidal capillaries in marrow of L6 vertebra, ⇔ arterial capillaries (Laroche <i>et al.</i> 1996). ↑ and ⇔ vasoconstriction in metaphyseal and diaphyseal arteries, respectively, from the femur, ⇔ vasodilation in metaphyseal and diaphyseal arteries (Hansen <i>et al.</i> 2001). ↓ mRNA of VEGF 120 and 164, ↓ endothelial and inducible NO synthase, ↓ fibroblast growth factor-2 in proximal tibia metaphysis at day 3 OVX, ↓ blood vessel number at day 7 OVX (Mekraldi <i>et al.</i> 2003). ↓ Endothelium-dependent relaxation and ↑ contraction in aorta and femoral arteries, ⇔ endothelium-independent relaxation in aorta and femoral artery, ↓ lumbar vertebral perfusion (Griffith <i>et al.</i> 2010). ↓ and ⇔ endothelium-dependent vasodilation in PNAs from young and old OVX rats, respectively, ⇔ endothelium-independent vasodilation in PNAs from both groups (Prisby <i>et al.</i> 2012). ↓ Bone marrow vascular density, ↑ tibia VR, ↓ tibia perfusion (Roche <i>et al.</i> 2013). ↓ Bone marrow vascular density and volume, ⇔ vessel size, ↑ tibia VR and ↓ perfusion (Roche <i>et al.</i> 2014).
Vitamin D	
Supplementation	<ul style="list-style-type: none"> ↑ Brachial flow-mediated dilation (Harris <i>et al.</i> 2011). ⇔ Brachial flow-mediated dilation (Gepner <i>et al.</i> 2012, Yiu <i>et al.</i> 2013). ↑ Relaxation of renal arteries from OVX rats with 12 h of calcitriol incubation, ↑ endothelium-dependent relaxation in renal arteries following supplementation in OVX rats (Dong <i>et al.</i> 2013).
Deficiency	<ul style="list-style-type: none"> ↓ Brachial flow-mediated dilation (Yiu <i>et al.</i> 2011). ↓ NO bioavailability and NO synthase 3 gene expression and protein content in VDR knockout mice (Andrukhova <i>et al.</i> 2014).
Calcitonin and CGrP	
Administered doses	<ul style="list-style-type: none"> ↓ Skeletal blood flow (Wootton <i>et al.</i> 1978). ↑ Perfusion pressure in isolated tibia (Driessens & Vanhoutte 1981). ⇔ In elevated tibia and femur blood flow induced by paraplegia, ↑ femur blood flow in intact rats (Schoutens <i>et al.</i> 1988). ↑ Relaxation in pre-constricted arterial segments (Lundgaard <i>et al.</i> 1997). ⇔ Marrow VR, ↓ tibia and femur marrow flow velocities, ↓ MAP (Vendéghe <i>et al.</i> 2010). ↑ Vasodilation in PNA (RD Prisby, unpublished observations).

↑, increase; ↓, decrease; ⇔, no change; E2, estrogen supplementation; L6, lumbar 6; MAP, mean arterial pressure; NO, nitric oxide; OVX, ovariectomy; PNA, principal nutrient artery; VDR, vitamin D receptor knockout mice; VEGF, vascular endothelial growth factor; VR, vascular resistance.

Conclusion

Mechanical perturbations such as exercise and hormones (e.g., PTH and estrogen), classically recognized as bone anabolic agents, have profound influences on the bone vasculature. Evidence suggests that these stimuli trigger modifications in bone blood vessels more rapidly than in bone. Further, inhibition of the vascular adaptation with these stimuli prevents or attenuates bone accrual. Bone blood vessels may mediate changes through the NO signaling pathway, particularly for bone adaptation in response to advancing age, mechanical loading such as exercise, PTH and estrogen. Interestingly, bone mass was strongly associated with endothelium-dependent vasodilator capacity of bone blood vessels and poorly associated with serum estrogen concentrations. While it is evident that other hormones such as vitamin D and calcitonin influence both bone and blood vessels, present data are too scarce to decipher the interplay between the two systems. [Table 2](#) provides a summary of how these osteogenic agents influence the bone vascular network.

The intimate bi-communication between bone blood vessels and bone is undeniable; however, these communications should not indicate mutual dependence between the systems. The vascular system can exist without bone, as evidenced by blood vessels in tissue beds outside of the skeleton. However, bone cannot form without the vascular system and healthy bone cannot exist without properly functioning blood vessels. Bone cells modulate vascular function via the release of growth factors and metabolic byproducts to enhance angiogenesis and bone blood flow. Yet, the *in vivo* modulation of bone cellular activity by neighboring blood vessels has not been thoroughly appreciated. For example, interstitial fluid flow and pressure is thought to be the mechanism by which mechanical loading imparts bone anabolic effects. Interstitial fluid flow and pressure are governed by the microcirculation. Besides controlling and directing blood flow, the microcirculation releases many factors involved in a variety of bone cellular activities. Thus, dysfunction of the microcirculation, which is known to occur with advancing age and malady, can contribute to bone and bone marrow pathology in a fashion independent of bone disease.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review. The content presented in this manuscript is solely the responsibility of the author and

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