Inhibition of vascular endothelial growth factor in young adult mice causes low bone blood flow and bone strength with no effect on bone mass in trabecular regions

N.E. Lanea,⁎, J.S. Nymanb, S. Uppugantib, A.J. Chaudharić, J.I. Aguirred, K. Shidaraa, X.P. Liua, W. Yaoa, D.B. Kimmelde

a Center for Musculoskeletal Health, University of California at Davis Medical Center, Sacramento, CA 95817, USA
b Department of Orthopaedic Surgery and Rehabilitation, Vanderbilt University Medical Center, Nashville, TN 37232, USA
c Center for Molecular and Genomic Imaging, Department of Radiology, University of California at Davis, Davis, CA 95616, USA
d Department of Physiological Sciences, University of Florida, Gainesville, FL 32610, USA

ARTICLE INFO

Keywords:
Anti-VEGF antibody
18F-NaF-PET/CT
Ultimate load
Bone mineral density (BMD)
Bone water
Trabecular
Cortical
Bone quality
Microarchitecture

ABSTRACT

Objective: To determine the effect of an antibody to vascular endothelial growth factor (VEGF) on bone blood flow, bone strength, and bone mass in the young adult mouse.

Methods: Ten-week-old male BALB/cJ mice were body weight-randomized into either a rodent anti-VEGF monoclonal antibody (anti-VEGF, B20-4.1.1; 5 mg/kg 2×/wk.; n = 12) group or a vehicle (VEH; n = 12) group. After 42 days, mice were evaluated for bone blood flow at the distal femur by 18F-NaF-PET/CT and then necropsied. Samples from trabecular and cortical bone regions were evaluated for bone strength by mechanical testing, bone mass by peripheral quantitative computed tomography (pQCT), and microarchitecture (MicroCT). Hydration of the whole femur was studied by proton nuclear magnetic resonance relaxometry (1H NMR).

Results: Distal femur blood flow was 43% lower in anti-VEGF mice than in VEH mice (p = 0.009). Ultimate load in the lumbar vertebral body was 25% lower in anti-VEGF than in VEH mice (p = 0.013). Bone mineral density (BMD) in the trabecular region of the proximal humeral metaphysis by pQCT, and bone volume fraction and volumetric BMD by MicroCT were the same in the two groups. Volume fraction of bound water (BW) of the whole femur was 14% lower in anti-VEGF than in VEH mice (p = 0.003). Finally, BW, but not cortical tissue mineral density, helped section modulus explain the variance in the ultimate moment experienced by the femur in three-point bending.

Conclusion: Anti-VEGF caused low bone blood flow and bone strength in trabecular bone regions without influencing BMD and microarchitecture. Low bone strength was also associated with low bone hydration. These data suggest that bone blood flow is a novel bone property that affects bone quality.

1. Introduction

Non-invasive assessment of fracture risk in humans involves measuring a series of bone properties that contribute to bone strength. DXA-based areal bone mineral density (BMD) of the spine and hip, a well-established endpoint that evaluates bone mass, has set a solid standard for evaluating prospective fracture risk in individuals (Kanis et al., 1994; Kanis and WHO Study Group, 1994). However, three independent lines of reasoning support the idea that by itself, BMD is insufficient to completely integrate all aspects of bone fragility. First, a history of fragility fracture at the spine or hip is a better predictor of future fracture than BMD (Ross et al., 1993). Second, anti-resorptive therapy with a nitrogen-containing bisphosphonate (N-BP) or a RANK Ligand antibody, that increases spine BMD ~6–10% and hip BMD ~3–4% after three years, is far more effective in reducing fracture risk at the spine (by ~70%) and at the hip (by ~50%), than the BMD increases predict (Cummings et al., 1998; Harris et al., 1999; Delmas and Seeman, 2004; Cummings et al., 2002). Third, glucocorticoid (GC)-treated, diabetic, and hyperhomocysteinemic patients have higher fracture risk than is predicted by their BMD (Van Staa et al., 2003; Balasubramanian et al., 2018; Saag et al., 2009; Weinstein et al., 2010; Sellmeyer et al., 2016; Rubin, 2017; Behera et al., 2017; Blouin et al., 2009). This BMD/fracture risk gap has led to the study of bone quality as a bone mass-independent contributor to fracture risk (Fonseca et al., 2016).
Bone properties that may influence fracture risk independent of bone mass can be assessed by various non-invasive imaging techniques (bone geometry, trabecular microarchitecture, and cortical microstructure) (Manhard et al., 2017), a minimally invasive indentation method (Chang et al., 2018); serum biomarkers of bone turnover, collagen cross-linking, and protein glycation (Garnero, 2012); and analysis of bone biopsies (bone matrix composition, collagen fiber orientation, and degree of bone mineralization (Roschger et al., 2014).

Since vascular homeostasis is critical to healthy bone, one might hypothesize that bone blood flow is another such bone property (Weinstein et al., 2011; Fonseca et al., 2014). Under poor local perfusion or ischemic conditions, new blood vessels form to restore normal oxygen saturation or, in the case of bone, an adequate state of hypoxia (4% O2). In healthy tissue, growth factors (e.g., fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF) act through paracrine or autocrine mechanisms to stimulate production of vascular endothelial growth factor (VEGF), which both maintains existing blood vessels and stimulates sprouting of new blood vessels (Senger, 2010; Murakami, 2012; Murakami and Simons, 2009; Maurea et al., 2016; Hu and Olsen, 2016a). New blood vessel formation in bone tissue is critical for vascular maintenance, healing bone defects, and even bone remodeling (Hu and Olsen, 2016b; Tabbaa et al., 2014). When that vasculization is impeded, blood perfusion and formation of collateral circulation slows (Murakami, 2012; Liu et al., 2014). When juvenile male rats were treated with a bone anabolic dose of PTH or PTH combined with a rodent anti-VEGF antibody (anti-VEGF) for six weeks, PTH + anti-VEGF rats had 4% lower BMD, but 13% lower maximum load and 37% lower bone toughness in the femoral diaphysis than rats treated with only PTH. The lower bone strength thus appeared disproportionate to lower BMD, suggesting that anti-VEGF inhibits the ability of PTH to improve bone strength as much as the bone mass effect of PTH would suggest (Rhee et al., 2009). When adult male rats were given a bone anabolic dose of PTH or PTH combined with bevacizumab (BVZ) for 15d, PTH increased bone mass and bone formation, but those changes were blunted by BVZ (Prisby et al., 2011; Prisby et al., 2013). Rats given BVZ monotherapy had fewer blood vessels, with a significant difference from control rats in skeletal phenotype (Prisby et al., 2011; Prisby et al., 2013). Despite the importance of vascularization to both bone homeostasis and the anabolic action of PTH, the effects of low bone blood flow on bone strength and bone mass have not been studied. Importantly, anti-VEGF monotherapy does not appear to significantly influence bone formation and bone mass in adult rats.

Numerous pre-clinical studies of bone-active agents show concurrent, correlated effects on both bone strength and bone mass (PTH, GCs, N-BPs, etc.) (Mohan et al., 2017; Li et al., 1991; Isowa et al., 2010; Allen et al., 2006). Occasional pre-clinical studies have evaluated agents that affect bone strength with minor effects on BMD (Allen et al., 2006). Based on what has been learned about the maintenance of vascular integrity by clinical use of VEGF inhibitors (Senger, 2010; Murakami, 2012; Murakami and Simons, 2009; Maurea et al., 2016; Hu and Olsen, 2016a), our approach was to treat adolescent/young adult mice with anti-VEGF, an agent that reduces bone blood vessel number (Prisby et al., 2011) and then study bone blood flow, bone mass and bone strength. We hypothesized that bone blood flow is associated with bone strength independently of bone mass. While this type of agent inhibits the maintenance of vascular integrity (Murakami, 2012; Murakami and Simons, 2009; Maurea et al., 2016; Kamba and McDonald, 2007; Fish and Wythe, 2015; Baffert et al., 2006; Zeb et al., 2007) and causes low blood flow, its effects on bone mass and bone strength are currently unknown.

2. Materials and methods

2.1. Animals

Nine-week-old male BALB/cJ (n = 24) mice (Jackson Laboratory; Sacramento, CA, USA) were purchased and housed for seven days at the UC Davis Animal Facility. Mice were kept singly in plastic cages with a 12:12-hour dark:light cycle and a temperature range of 20–22 °C. They were fed commercial rodent chow (22.5 Rodent Diet; Teklad; Madison, WI) ad libitum. After one week, mice were body weight-randomized into either a rodent anti-VEGF monoclonal antibody group (anti-VEGF; B20-4.1.1; Genentech; South San Francisco, CA) (n = 12) (Tegnebratt et al., 2018; Jiang et al., 2014) or a vehicle group (VEH; n = 12; phosphate-buffered saline). Mice were then treated twice weekly for six weeks with anti-VEGF (5 mg/kg; 0.1 cc/100 g BW) or VEH by subcutaneous injection ( Wu et al., 2012). Body weight was measured weekly. The study was carried out following recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health with the approval of the UC Davis Institutional Animal Care and Utilization Committee.

2.2. Bone blood flow measurement (in vivo 18F-NaF-post positron emission tomography/computed tomography (18F-NaF-PET/CT) imaging)

Mice were anesthetized using isoflurane/O2 (3% induction, 1.5–2% maintenance) for a total of 55 min. Anesthetized mice were secured on a portable scanning bed of a small-animal PET scanner (Inveon DPET, Siemens Healthcare; Knoxville, TN, USA) (Bao et al., 2009). The PET acquisition was initiated and 18F-Nafluoride (~9.25 MBq) was injected intravenously via a tail vein catheter. Dynamic imaging was performed and listmode data were collected for 45 min. After the PET scan was complete, the scanning bed with the mouse still secured was moved to an adjacent small-animal computed tomography (CT) scanner (Inveon, Siemens Healthcare; Knoxville, TN, USA), where a whole-body CT scan was performed. External fiducial markers on the scanning bed guided the subsequent registration of the PET and CT images. The PET dynamic data were reconstructed into 43 time frames (12 × 10 s, 16 × 30 s, 10 × 60 s, 5 × 300 s) using the manufacturer’s software. Analysis of 18F-Na uptake kinetics was carried out using the PMOD software (PMOD 3.802; PMOD Technologies Ltd.; Zurich, SW) of which the first 33 (15 min) were used. First, ellipsoidal volumes of interest (VOIs) for each distal femur were manually drawn on the PET image, which the

![Fig. 1. Representative 18F-NaF PET/CT maximum intensity projection image of lower half of mouse body during 0–15 min post 18F-NaF injection. Femurs, tibiae, lower spine, and feet are visible from CT scan (grayscale). Red-orange spots represent 18F-NaF PET signal location. Note ellipsoidal VOI over distal femur. White area in center is bladder. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
needed for compartmental modeling was derived from the images of the left ventricle of the heart. The primary rate constant derived for each VOI was $k_0$, the forward capillary transport parameter. $k_1$ (ml/cc/min) is the product of bone blood flow and the $^{18}$F-NaF extraction fraction (Czernin et al., 2010). Because of the high single-passage extraction of $^{18}$F-NaF (Wootton and Doré, 1986), $k_1$ correlates strongly with bone blood flow (Piert et al., 1998; Piert et al., 2001; Temmerman et al., 2008; Ottley et al., 2017; Chen et al., 2015). In each mouse, $k_1$ was derived separately for each distal femur.

2.3. Necropsy

$^{18}$F-NaF PET/CT scanning was conducted the day before necropsy. At necropsy, mice were euthanized by CO$_2$ asphyxiation followed by cardiac puncture. Cervical dislocation was used as a secondary method of euthanasia. Both femurs were dissected free at the acetabulum and separated from the tibias. Lumbar vertebra 3–6 were dissected free from the other vertebrae. Both humeri were dissected free from the scapula and forelimb bones. All bones were cleansed gently of attached muscle, wrapped in saline-soaked gauze, and frozen at −20 °C.

2.4. Biomechanical testing

2.4.1. Lumbar vertebral body 6 (compression)

The L6 vertebral body (LVB6), a trabecular bone rich site, was mechanically tested in axial compression. Specimens were thawed at room temperature for two hours. Posterior elements were trimmed from each LVB6 at the pedicle, and endplates were cut parallel using a low-speed, water-irrigated, circular bone saw (Isomet 1000; Buehler, Lake Forest, IL), such that the test specimens had a longitudinal axis length of ~1.5 mm. Specimens were rehydrated with phosphate-buffered saline prior to testing, and were kept hydrated during testing. Samples were affixed to loading platens with cyanoacrylate glue, then compressed to failure at 0.05 mm/s using an electromagnetic materials testing system (Bose ELF 3200; San Diego, CA USA). Customary variables relating to strength, yield stress, and work to failure.

2.4.2. Femoral diaphysis (three-point bending) Three-point bending tests of hydrated right femurs were performed using a servohydraulic material testing system (Instron DynaMight 8841; Norwood, MA) fitted with a linear variable displacement transducer (attached to the actuator) and a 100 N load cell (Honeywell; Columbus, OH; Model # 060-C863-02). Specimens were thawed at room temperature for two hours. The specimens were placed anterior surface down and medial side forward, and then loaded to failure at the midpoint, at 3 mm/min with a fixed lower span of 8 mm. One femur from the VEH group could not be stabilized during testing, leading to the exclusion of that bone’s three point bending test data (see below). Customary whole-bone structural properties were determined from the force vs. displacement curve (Makowski et al., 2014; Nyman et al., 2011a; Nyman et al., 2011b; Turner and Burr, 1993). These included ultimate load, yield load, stiffness, yield stress, and work to failure.

2.5. Areal bone mass measurement (peripheral quantitative computed tomography (pQCT)) pQCT of the right humerus was performed to determine areal bone mass in trabecular and cortical bone regions. The humerus was scanned in 1 mm thick cross sections using a Stratec XCT Research M instrument (v5.40, Norland Medical Systems; Fort Atkinson, WI). The sites of interest were a 1 mm slice centered at 5 mm distal to the proximal end of the humerus (proximal metaphysis), and a 1 mm slice centered at the longitudinal midpoint of the humerus (mid-diaphysis or central region), Bone mineral content (BMC, g), bone area (Ar, cm$^2$), and areal bone mineral density (RMD, mg/cm$^2$) were determined for trabecular bone of the proximal humeral metaphysis and cortical bone of the central femur (Brodt et al., 2003; Schmidt et al., 2003).

2.6. $^1$H-nuclear magnetic resonance relaxometry (NMR)

Prior to the three-point bending test, volume fractions of bound water (BW), pore water (PW), and solid proton, were measured in the intact right femur using $^1$H NMR relaxometry (Horch et al., 2011a). After thawing to room temperature, the femur was blotted to remove surface water and placed in a capped NMR-tube with 20 μL of deionized water in sealed glass (reference marker with a long $T_2$) = ~2.5 s). An in-house-built radio frequency (RF) coil with very low background proton signal (Horch et al., 2010) was used for excitation and signal reception. Upon placing the coil in a 4.7 T horizontal bore magnet (Varian Medical Systems, Santa Clara, CA), Carr-Purcell-Meiboom-Gill (CPMG) measurements with a total of 10,000 echoes were acquired at an echo spacing of 100 μs. The CPMG data vector was fitted to 256 decaying exponentials with time constants between 100 μs and 10 s and subject to non-negative and minimum curvature constraints to produce a $T_2$ spectrum (Fig. 3). The area of signal was integrated for three $T_2$ spectral components (0.172 ms to 0.65 ms for BW, 0.65 ms to 42 ms for PW, and 760 ms to 10 s for the reference marker). BW and PW areas were converted to volume using the ratio of the reference marker area to the known water volume, and then divided by the bone specimen volume measured by Archimedes’ principle, to obtain BW and PW as a percentage.

2.7. Microcomputed tomography (MicroCT)

The right distal femoral metaphysis (DFM) and sixth lumbar vertebra (L6V) were imaged ex vivo using a high-resolution scanner (VivoCT 40, Scanco Medical AG, Bassersdorf, Switzerland) at 70 kVp and 145 μA with scan settings that provided an isotropic voxel size of resolution of 10.5 μm in all three dimensions. Scanning of the DFM was initiated 1 mm proximal to the distal end of the bone and extended proximally for 250 slices. Scanning of L6V was initiated at its caudal end and continued to its cranial end. 3D reconstruction of each scan was done with manufacturer’s software. For the DFM, a secondary spongyosa VOI with a proximal boundary 0.2 mm proximal to the most proximal point of the growth cartilage-metaphyseal junction (GCMJ) and extending 150 slices proximal, was used. For L6V, the trabecular bone region of the body was analyzed. A VOI that started 0.1 mm cranial to the caudal GCMJ and ended 0.1 mm caudal to the cranial GCMJ was used. A trabecular-cortical boundary at 0.15 mm from the endocortical surface was established for each slice. All trabecular bone inside the boundary was evaluated. The methods for calculating bone volume (BV), total volume (TV), connectivity density, volumetric trabecular bone mineral density (vTb.BMD), trabecular tissue mineral density (Tb.TMD), trabecular thickness (Tb.Th), trabecular number (Tb.N), and structure model index (SMI) have been described (Bouxsein et al., 2010).

The right femoral diaphysis was imaged as above. Scanning was initiated 0.5 mm proximal to the mid-point of the bone and extended distally for 100 slices. From beam theory, the ultimate moment is directly proportional to the cross-sectional geometry factor, section modulus ($I_{min}/c_{min}$), where $I_{min}$ is the moment of inertia for bending within the anterior-posterior plane and $c_{min}$ is the distance between neutral axis (zero stress) and outermost bone surface in the direction of loading. $I_{min}/c_{min}$ was calculated using Slice 51 of the scan.

2.8. Statistical analysis

The group means and standard deviations were calculated for all variables (GraphPad Prism 7.00 (GraphPad Software, La Jolla, CA).
USA). The Mann-Whitney \( U \) test was used to assess intergroup differences. Differences were considered significant at \( p < 0.05 \).

Pearson’s correlation coefficient for end of study body weight to bound water and pore water was calculated separately for the VEH and anti-VEGF groups.

To determine whether the structural-dependent bending strength (ultimate moment) of the central femur was solely explained by the cross-sectional geometry (section modulus) or helped by other properties of the femur (that differed between treatment groups), linear regressions were performed using general linear models [Stata 11.0, (StataCorp; College Station, TX USA)] in which the interaction term was excluded if not significant (\( p > 0.05 \)).

3. Results

3.1. Pre-necropsy body weight and bone blood flow (Table 1, Fig. 2A)

Final body weight was 10% lower (\( p = 0.001 \)) in anti-VEGF-treated mice than in VEH-treated mice. Distal femoral blood flow, as measured by \( K_1 \), at both the right and left sides was 43% lower (\( p = 0.009 \)) in anti-VEGF-treated than in VEH-treated mice (Fig. 2A).

3.2. Trabecular and cortical bone strength (Table 2, Fig. 2B)

Ultimate load of LVB6 was 25% lower (Fig. 2B, \( p = 0.013 \)) and stiffness was 44% lower (Table 2, \( p = 0.044 \)) in anti-VEGF-treated mice than in VEH-treated mice. Work to failure at LVB6 was not a ffected by anti-VEGF. Though ultimate load at the central femur was 8% lower (\( p = 0.032 \)) in anti-VEGF-treated than in VEH-treated mice, all other biomechanical properties at the central femur were the same in the two groups (Table 2).

3.3. Bone mass of humerus (Table 1, Fig. 2C)

Metaphyseal trabecular BMC, BMD (Fig. 2C), and bone area were the same in anti-VEGF-treated and VEH-treated mice. Cortical BMD was 1.9% higher (\( p = 0.045 \)) and cortical thickness was 6.1% higher (\( p = 0.024 \)) in anti-VEGF-treated mice than in VEH-treated mice (Table 1). Cortical BMC and cortical area also trended higher in anti-VEGF-treated mice than in VEH-treated mice (Table 1).
3.4. Bone water endpoints (Table 2, Fig. 2D)

Volume fraction of bound water of the left femur was 14% lower (Fig. 2D, p = 0.003) in anti-VEGF-treated than in VEH-treated mice. No other NMR properties were affected by anti-VEGF (Table 2). There was no significant correlation of volume fraction of bound water or pore water to ending body weight (Table 4).

3.5. Microarchitecture (Table 3)

Connectivity density in the lumbar vertebral body was 11% higher (p = 0.028) and trabecular thickness was 7% lower (p = 0.028) in anti-VEGF-treated mice than in VEH-treated mice (Table 3). No other microarchitectural endpoints in the lumbar vertebral body were affected by anti-VEGF.

At the distal femoral metaphysis, SMI was 11% higher (p = 0.024) and trabecular thickness was 21% higher (p = 0.002) in anti-VEGF-treated than in VEH-treated mice (Table 3). However, no other microarchitectural endpoints were affected by anti-VEGF. Tissue mineral density of trabecular bone (Tb.TMD) was 7% higher (p = 0.001) in anti-VEGF-treated than in VEH-treated mice (Table 3).

No structural endpoints at the central femur were significantly affected by anti-VEGF. Tissue mineral density of cortical bone (Ct.TMD) was 1.4% lower (p = 0.011) and volumetric bone mineral density (Ct.BMD) was 1.5% lower (p = 0.007) in anti-VEGF-treated than in VEH-treated mice (Table 3).

![Fig. 3. Assessment of bone water (1H-nuclear magnetic resonance relaxometry (NMR)).](image)

Representative relaxation time constant ($T_2$) spectra from femurs of anti-VEGF (blue) and VEH (black) mice. Normalized intensity (y-axis) vs. log Relaxation Time (sec) (x-axis) The solid protons involved in covalent bonds (Solid Proton Band with peak near $T_2 = 10^{-4}$ s) relax faster than the water protons participating in hydrogen bonding within the organic matrix (Bound Water Band with peak near $T_2 = 10^{-4}$ s). The water protons participating in hydrogen bonding with this organic matrix in turn relax faster than the water protons residing in pores (Short T2 Pore Water bands ranging between $T_2 = 10^{-4}$ s and $T_2 = 4 \times 10^{-2}$ s). The reference marker is a known volume of water. The Reference Marker thus has the slowest relaxation (i.e., longest $T_2$ values between 1 and 10s).

Because marrow was present, the area under the pore water signals was integrated between the two vertical lines spanning $6.5 \times 10^{-4}$ s $< T_2 < ~4 \times 10^{-2}$ s. Therefore, this measurement not only excluded water in large pores, but also avoided the lipid signal. Both the area of the Bound Water Band and the area of the Pore Water Band were converted to the volume of water using the area under the Reference Marker band.

Note that all peaks for the representative $T_2$ spectrum from the anti-VEGF mouse are lower than those for the VEH mouse. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### Table 1

| Endpoint                      | Vehicle | Anti-VEGF |
|-------------------------------|---------|-----------|
| Final body weight g           | 12      | 12        |
| Blood flow ml/cc/min          | 6       | 6         |
| Humerus bone mass             |         |           |
| Proximal metaphysis           |         |           |
| Trabecular BMC mg             | 10      | 11        |
| Trabecular area cm$^2$        | 12      | 12        |
| Diaphysis                     |         |           |
| Cortical BMC mg               | 12      | 12        |
| Cortical BMD mg/cm$^2$        | 12      | 12        |
| Cortical area cm$^2$          | 12      | 12        |
| Cortical thickness mm         | 12      | 12        |

BMC- bone mineral content.  
BMD- bone mineral density.  
$p = \text{Mann-Whitney U.}$  

### Table 2

| Endpoint                        | Vehicle | Anti-VEGF |
|---------------------------------|---------|-----------|
| Lumbar vertebral body 6 (compression) (trabecular) Stiffness N/mm | 12  168.6 ± 97.3 | 11  105.0 ± 53.7 |
| Work to failure J               | 12  8.69 ± 4.75 | 11  6.66 ± 3.19 |
| Right central femur 3 point bending (cortical) Ultimate load N | 12  24.58 ± 1.72 | 11  22.69 ± 2.03 |
| Yield load N                    | 11  19.49 ± 1.70 | 12  18.48 ± 2.10 |
| Stiffness N/mm                  | 11  142.8 ± 12.7 | 12  137.4 ± 13.4 |
| Yield stress N/mm$^2$           | 11  178.2 ± 20.9 | 12  179.2 ± 17.0 |
| Work to failure J               | 11  5.56 ± 0.82  | 12  5.80 ± 1.06 |
| Solid proton %                  | 12  3.92 ± 0.42  | 12  3.86 ± 0.51 |
| Pore water volume fraction %    | 12  15.49 ± 1.19 | 12  16.81 ± 3.44 |

$p = \text{Mann-Whitney U.}$

was 1.4% lower (p = 0.011) and volumetric bone mineral density (Ct.BMD) was 1.5% lower (p = 0.007) in anti-VEGF-treated than in VEH-treated mice (Table 3).
Table 3  
Microarchitecture of vertebral body and left femur.

| Endpoint                     | Vehicle | anti-VEGF |
|------------------------------|---------|-----------|
|                              | Units   | N         | Mean ± SD | N         | Mean ± SD | p =         |
| Lumbar vertebral body 6      | %       | 9         | 26.8 ± 2.2 | 11        | 25.0 ± 2.7 | 0.074      |
| BV/TV                        |         |           |           |           |           |            |
| Connectivity density         | mm⁻²    | 9         | 230.2 ± 33.0 | 11        | 260.6 ± 25.2 | 0.028      |
| SMI                         |         | 9         | 0.370 ± 0.255 | 11        | 0.496 ± 0.269 | 0.277      |
| Trabecular number            | mm⁻¹    | 9         | 4.96 ± 0.16 | 11        | 4.96 ± 0.38 | 0.590      |
| Trabecular thickness         | mm      | 9         | 0.053 ± 0.003 | 11        | 0.049 ± 0.003 | 0.028      |
| Volumetric Tb.BMD            | mgHA/cm³ | 12       | 209.4 ± 26.9 | 12        | 189.8 ± 31.2 | 0.117      |
| Tb.TMD                      | mgHA/cm³ | 12       | 722 ± 17 | 12        | 722 ± 20 | 0.977      |
| Left distal femur            | %       | 12        | 157.5 ± 3.2 | 12        | 16.9 ± 2.8 | 0.242      |
| BV/TV                        |         |           |           |           |           |            |
| Connectivity density         | mm⁻²    | 12        | 188.7 ± 72.5 | 12        | 133.4 ± 32.1 | 0.060      |
| SMI                         |         | 12        | 2.02 ± 0.34 | 12        | 2.23 ± 0.22 | 0.024      |
| Trabecular number            | mm⁻¹    | 12        | 4.92 ± 0.69 | 12        | 4.77 ± 0.32 | 0.378      |
| Trabecular thickness         | μm      | 12        | 46.9 ± 4.1 | 12        | 56.9 ± 9.0 | 0.002      |
| Volumetric Tb.BMD            | mgHA/cm³ | 12       | 160.5 ± 27.9 | 12        | 175.2 ± 20.3 | 0.127      |
| Tb.TMD                      | mgHA/cm³ | 12       | 724 ± 15 | 12        | 774 ± 21 | 0.001      |
| Left central femur           | %       | 11        | 1.031 ± 0.062 | 12        | 0.988 ± 0.053 | 0.169      |
| Cortical area                | mm³     |           |           |           |           |            |
| Total area                   | mm³     | 11        | 1.751 ± 0.120 | 12        | 1.712 ± 0.109 | 0.525      |
| Moment of inertia            | mm⁴     | 11        | 0.135 ± 0.017 | 12        | 0.123 ± 0.015 | 0.089      |
| Cortical area/total area     | %       | 11        | 58.9 ± 1.8 | 12        | 57.8 ± 1.2 | 0.136      |
| Cortical thickness           | mm      | 11        | 0.250 ± 0.013 | 12        | 0.247 ± 0.006 | 0.695      |
| Volumetric Ct.BMD            | mgHA/cm³ | 11       | 1065 ± 11 | 12        | 1053 ± 21 | 0.032      |
| ClTMD                        | mgHA/cm³ | 12       | 1091 ± 12 | 12        | 1078 ± 11 | 0.037      |

p = Mann-Whitney U.  
TMD- tissue mineral density.  
HA-hydroxyapatite.

3.6. Multivariate explanation of strength

From beam theory, the ultimate moment applied to a femur is directly proportional to the section modulus of the mid-shaft (i.e., \( I_{\text{min}}/c_{\text{min}} \)). Section modulus explained 63.9% of the variance in ultimate moment (Fig. 4). Standardized coefficients (β) for explanatory variables indicated that BW (p = 0.007) helped section modulus explain the variance in ultimate moment, whereas Ct.TMD of the diaphysis was not useful (Table 5).

4. Discussion

Using an anti-VEGF antibody to inhibit blood flow, the present study may have established an association between bone blood flow and trabecular bone quality. Both blood flow, assessed by in vivo ¹⁸F-NaF-PET/CT of the distal femur, and bone strength, assessed by an ex vivo compression test of LBV6, were lower in anti-VEGF-treated mice than in VEH-treated mice that showed no treatment-related differences in bone mass. Specifically, BMD and BMC of the proximal humeral metaphyseal trabecular region, as well as bone volume fraction and volumetric BMD of the distal femoral metaphysis and LBV6, were not affected by anti-VEGF, while ultimate load, the principle indicator of whole bone strength, was 25% lower in LBV6 of anti-VEGF-treated mice than in VEH-treated mice. Therefore, low bone blood flow appears to cause low bone strength without causing low bone mass, indicating that it may reduce bone strength through an effect on bone quality. These data support our hypothesis that bone blood flow is associated with bone strength independently of bone mass.

Our results parallel data from F344 rats, in which an age-related decrease in bone blood flow was associated with lower ultimate bending stress of the central femur despite higher femoral BMD (Bloomfield et al., 2002). An experiment that applies a second class of anti-angiogenic agents, such as tyrosine kinase inhibitors (Lopez et al., 2019; Bai and Zhang, 2018), would be necessary to reveal whether low bone blood flow itself, rather than an intrinsic effect of this anti-VEGF antibody, causes low bone strength. In any case, our data indicate that bone blood flow is positively associated with whole bone strength with no effect on bone mass in red marrow trabecular bone rich regions (distal femoral metaphysis, LBV6, and proximal humeral metaphysis), suggesting that bone blood flow is a bone property that influences bone quality.

VEGF supports vascular integrity in all organs by both maintaining the vasculature and stimulating angiogenesis (Senger, 2010; Murakami, 2012; Murakami and Simons, 2009; Maurea et al., 2016; Hu and Olsen, 2016a). We chose anti-VEGF with the aim of improving the understanding of sources of excessive bone fragility in GC-induced osteoporotic (GIOP) patients. Unlike GCs that reduce both BMD and bone blood flow (Weinstein et al., 2010; Mohan et al., 2017), we envisioned anti-VEGF as a bone mass-neutral agent that would act as a biological tool that specifically reduces bone blood flow, allowing direct studies of...
the effect of low blood flow on bone strength, without the uncertainty introduced by the negative BMD effects of GCs that themselves decrease bone strength.

Identifying a physical property of bone tissue that is positively-related to both bone blood flow and bone strength could improve the current understanding of how bone blood flow affects bone strength. Therefore, we measured volume fraction of bound water (BW), the amount of water interacting with the bone matrix, in the whole femur, a property known to be related to bone strength (Nyman et al., 2006). While anti-VEGF had no significant effect on bone mass and microarchitectural properties of trabecular bone regions of the humerus and femur, anti-VEGF mice were lower in VEH-treated mice. Importantly, there was no correlation of BW endpoints to study body weight in either treatment group (Table 4). Both low bone blood flow and volume fraction of BW are thus associated with low bone strength. Volume fraction of BW also significantly helped the bone geometry factor related to bending explain ultimate moment in cortical bone (Table 5). This suggests that clinical assessment of BW by MRI (Manhard et al., 2017) could improve the condition of these patients, suggesting that low bone blood flow explains a portion of their excess bone fragility. Half of all fragility fractures occur in individuals with osteopenia (BMD T-score < −1.0, but > −2.5), rather than osteoporosis (T-score < −2.5) (Kanis et al., 1994; Fonseca et al., 2014). As with GC and diabetic patients, studies of bone blood flow in BMD-matched osteopenic patients who have (or have not) experienced fragility fractures, could be used to examine the relationship of bone blood flow to excessive bone fragility (Rizzo et al., 2018; Polly et al., 2012).

Anti-VEGF (e.g., bevacizumab) is an FDA-approved adjuvant cancer treatment that reduces tumor angiogenesis and increases progression-free survival in colorectal, ovarian, and cervical cancer; and clear cell renal and non small cell lung carcinoma patients. Its documented adverse effects include hypertension, bleeding, abnormal blood clotting, myocardial infarction, cerebral vascular accident, inhibition of wound healing, and intestinal perforation (Fuca et al., 2018; Raouf et al., 2018; Chen et al., 2006; Lee et al., 2018). All potentially indicate mechanism-based, negative effects on vasculature of the non-cancerous portions of the body (Kamba and McDonald, 2007; Fish and Wythe, 2015; Baffert et al., 2006; Zeb et al., 2007). Our data suggest that it is reasonable to think that low bone blood flow may cause increased fracture risk not only in GC-treated subjects or in patients with diabetes, peripheral vascular disease, or osteopenia, but also in patients treated with anti-angiogenic agents. All these diseases and medications would likely express their negative effects on bone strength in humans as increased fracture risk not mediated through BMD, making trials that measure BMD without monitoring fracture uninformative.

These data may also provide additional information about how the

### Table 4
Pearson correlation coefficients of ending body weight to bone water variables.

| Variable                        | Group       | R   | p    | N  |
|---------------------------------|-------------|-----|------|----|
| Volume fraction of bound water  | VEH         | 0.3966 | 0.2018 | 12 |
| Volume fraction of pore water   | VEH         | 0.1195 | 0.7114 | 12 |
| Volume fraction of bound water  | Anti-VEGF   | 0.2341 | 0.4640 | 12 |
| Volume fraction of pore water   | Anti-VEGF   | 0.0127 | 0.9688 | 12 |

**β** = 0.799 (p < 0.0001) (p = 0.671) Cl.Th  
β = 0.730 (p < 0.0001) (p = 0.642) Cl.TMD  
β = 0.779 (p < 0.0001) (p = 0.591) BW  
β = 0.746 (p < 0.0001) (p = 0.586) BW

*BW* = volume fraction of bound water.  
NI = no interaction.

### Table 5
General linear models for ultimate moment.

| Covariate | Coefficient | Interaction | Adj R² (%) |
|-----------|-------------|-------------|------------|
| Cl.Th     | β = 0.261 (p = 0.052) | Ni | 66.7 |
| Cl.TMD    | β = 0.21 (p = 0.137) | Ni | 64.4 |
| BW        | β = 0.341 (p = 0.007) | Ni | 72.8 |
actions of existing pharmaceutical treatments for osteoporosis reduce fracture risk. Teriparatide (PTH) is a bone anabolic agent that reduces fracture risk much more than its effects on areal BMD would indicate (Chen et al., 2006). Our data suggest that part of that discrepancy might be explained by a positive effect of PTH on bone blood flow. Though some data indicate that PTH enhances blood flow through VEGF-dependent mechanisms, the findings are not universal. PTH restores endothelium dependent vasodilation in adult rats to the level in young rats (Lee et al., 2018) through activation of nitric oxide and protein kinases A and C (PKA and PKC) signaling (Benson et al., 2016). PTH-related vasodilatation of bone arteries appears dependent upon VEGF signaling within the vascular endothelium (Prisby et al., 2013; Isowa et al., 2010). PTH increases the release of VEGF from osteoblasts and osteocytes to stimulate new blood vessel formation (Isowa et al., 2010). PTH knockout mice display delayed fracture healing associated with reduced VEGF production from bone marrow stem cell derived osteoblasts (Ding et al., 2018). On the other hand, though PTH improves the vasodilatory capacity of blood vessels, it decreases blood vessel number in trabecular bone regions (Prisby et al., 2011). Though controversy exists concerning the effects of PTH on bone vasculature, our data might point to trials that study bone blood flow in teriparatide patients, to determine if positive bone blood flow effects of teriparatide explain a portion of its anti-fracture efficacy.

Some shortcomings of this experiment should be noted. Though we evaluated bone blood flow in a trabecular rich bone region using 18F-NaF PET/CT, we were unable to evaluate bone blood flow in a cortical bone region, because of the intrinsic spatial resolution limitations of PET and/or the low vascularity of cortical bone. Efforts are underway to overcome this problem using partial volume correction methods (Soret et al., 2007) and terminal 18F-NaF PET/CT scans that permit higher radiation doses that could better visualize regions with low blood flow. We measured bone blood flow, bone mass, and bone strength in three different trabecular bone rich sites (distal femur or distal femoral metaphysis, lumbar vertebral body, and proximal humeral metaphysis, respectively). The distal femur and proximal humerus are from the appendicular skeleton, while the lumbar vertebral body is from the axial skeleton. The three sites were chosen in spite of their location in different skeletal regions, because they are red marrow trabecular bone sites that display similar responses to currently prescribed bone-active agents. One should recognize that their individual responses to anti-VEGF may differ and were not evaluated here by traditional histomorphometric methods. We were unable to measure bone blood flow in an anatomical site that expresses increased bone fragility in osteoporotic subjects (i.e., LVB6), because the 18F-NaF PET signal in LVB6 cannot be separated from signal in overlying soft tissue or surrounding cortical portions of the whole vertebra. We were unable to measure bone water in trabecular bone regions. In future experiments, it should be possible to use pQCT to measure BMC and BMD in LVB6 before mechanical testing, distal femur BMC and BMD after 18F-NaF PET/CT, and central femur BMC and BMD where 18F-NaF PET/CT may eventually be possible. Administration of anti-VEGF began when mice were age 10 weeks, a time when bone elongation is slowing and the skeleton is best considered adolescent. Future studies that aim to interrogate the age at which the skeleton is considered mature adult.

5. Conclusions

Six weeks treatment of young adult mice with anti-VEGF appears to have caused low bone blood flow, bone strength, and bound water with no effect on bone mass. These data may suggest that bone blood flow is a novel bone property that affects bone quality.

Author statement

The following individual contributed, respectively to: study design/ planning (DBK, NEL), experimental conduct (WY, XPL, KS); data collection (JSN, SU, AJC, KS, XPL, JIA, WY); data analysis (JSN, DBK, AJC), original draft (DBK, NEL); revised draft (NEL, JSN, AJC, JIA, DBK).

Acknowledgments

This work was funded by National Institutes of Health Grants #s R01 AR043052-07, 1 P50 AR03043, P50 AR060752NII to NEL; and R21 AR072483 to JSN; the endowment for aging research at UC Davis to NEL; and the Center for Musculoskeletal Health at UC Davis.

Declaration of Competing Interest

Authors declare that they have no conflicts of interest in connection with this study.

Transparency document

The Transparency document associated with this article can be found, in online version.

References

Allen, M.R., Iwata, K., Sato, M., Burr, D.B., 2006. Raloxifene enhances vertebral mechanical properties independent of bone density. Bone 39, 1130–1135. https://doi.org/10.1016/j.bone.2006.05.007.
Baffert, F., Le, T., Seminno, B., et al., 2006. Cellular changes in normal blood capillaries undergoing regression after inhibition of VEGF signaling. Am Jour Physio 290, HS47-HS59.
Bai, Z.G., Zhang, Z.T., 2018. A systematic review and meta-analysis on the effect of anagenesis blockade for the treatment of gastric cancer. Oncos Targets Ther 11, 7077–7087. https://doi.org/10.2147/OTT.S169484. (eCollection 2018).
Balasubramanian, A., Wade, S.W., Adler, R.A., Saag, K., Pannaccione, N., Curtis, J.R., 2018. Glucocorticoid exposure and fracture risk in a cohort of US patients with selected conditions. J Bone Miner. Res. 33 (10), 1881–1888. https://doi.org/10.1002/jbmr.3523.
Bao, Q., Newport, D., Chen, M., Stout, D.B., Chatzizonnou, A.F., 2009. Performance evaluation of the In Vivo dedicated PET preclinical tomograph based on the NEMA NU4 standards. Jour Nucl Med 50 (3), 401–408. https://doi.org/10.2967/jnumed.108.056374.
Beckman, J.A., Creager, M.A., 2016. Vascular complications of diabetes. Circ. Res. 118 (11), 1771–1785. https://doi.org/10.1161/CIRCRESAHA.115.306684.
Behera, J., Bala, J., Nuru, M., Tyagi, S.C., Tyagi, N., 2017. Homocysteine as a pathological biomarker for bone disease. J. Cell. Physiol. 232 (10), 2704–2709. https://doi.org/10.1002/jcp.25693.
Benson, T., Menezes, T., Campbell, J., Rice, A., Hood, B., Prisby, R., 2016. Mechanisms of vasodilatation to PTH (1-84), PTH (1-34), and PTHrP (1-34) in rat bone resistance arteries. Osteoporos. Int. 27 (3), 1817–1826.
Bloomfield, S.A., Hogan, H.A., Delp, M.D., 2002. Decreases in bone blood flow and bone material properties in aging Fischer-344 rats. Clin Orthol Rel Res 396, 248–257.
Blouin, S., Thaler, H.W., Korninger, C., Schmid, R., et al., 2009. Bone matrix quality and plasma homocysteine levels. Bone 44 (5), 959–964. https://doi.org/10.1016/j.bone.2008.12.025.
Bouxsein, M.L., Boyd, S.K., Christiansen, B.A., Guldberg, R.E., Jepsen, K.J., Muller, R., 2010. Guidelines for assessment of bone microstructure in rodents using MicroCT. J Bone Miner Res 25, 1468–1466.
Briot, K., 2018. Bone and glucocorticoids. Ann Endocrinol (Paris) 79 (3), 115–118. https://doi.org/10.1016/j.jando.2018.04.016.
Brodt, M.D., Pelz, G.B., Taniguchi, J., Silva, M.J., 2003. Accuracy of peripheral quantitative computed tomography (pQCT) for assessing area and density of mouse cortical bone. Calcif. Tissue Int. 73 (4), 411–418.
Chang, A., Eason, G.W., Tang, S.Y., 2018. Clinical measurements of bone tissue mechanical behavior using reference point indentation. Clin Rev Bone Miner Metab 16, 87–94. https://doi.org/10.1016/j.crbmm.2018-01-0249-9.
Chen, P., Miller, P.D., Delmas, P.D., Mioussi, D.A., Krege, J.H., 2006. Change in lumbar spine BMD and vertebral fracture risk reduction in teriparatide-treated postmenopausal women with osteoporosis. J Bone Miner. Res. 21 (11), 1785–1790 Nov.
Chen, Y.J., Rosario, B.L., Mowrey, W., Laymon, C.M., Lu, X., Lopez, O.L., et al., 2015. Relative 11C-PiB delivery as a proxy of relative CBF: quantitative evaluation using single-session 15O-water and 11C-PiB PET. J. Nucl. Med. 56, 1199 ± 205. https://doi.org/10.2967/jnumed.114.152455. PMID: 26045309.
Cummings, S.R., Black, D.M., Thompson, D.E., et al., 1998. Effect of alendronate on risk of fracture in women with low bone density but without vertebral fractures: results from the fracture intervention trial. JAMA 280, 2077–2082.
Cummings, S.R., Karpath, D.B., Harris, F., Genant, H.K., Ensrud, K., et al., 2002. Improvement in spine BMD and reduction in risk of vertebral fractures during treatment with antiresorative drugs. Am. J. Med. 112, 281–289.
Tyagi, N., Vacek, T.P., Fleming, J.T., Vacek, J.C., Tyagi, S.C., 2011b. Hyperhomocysteinemia decreases bone blood flow. Vasc. Health Risk Manag. 25, 31–35. https://doi.org/10.2147/VHRM.S15844.

Van Staa, T.P., Laan, R.F., Barton, I.P., Cohen, S., Reid, D.M., Cooper, C., 2003. BMD threshold and other predictors of vertebral fracture in patients receiving oral glucocorticoid therapy. Arthritis Rheum. 48 (11), 3224–3229.

Weinstein, R.S., Wan, C., Liu, Q., Wang, Y., Almeida, M., et al., 2010. Endogenous glucocorticoids decrease skeletal angiogenesis, vascularity, hydration, and strength in aged mice. Aging Cell 9 (2), 147–161. https://doi.org/10.1111/j.1474-9726.2009.00545.

Wootton R, Doré C. The single-passage extraction of $^{18}$F in rabbit bone. Clin. Phys. Physiol. Meas.. 1986; 7(4):333–43.

Wu, F., Tamhane, M., Morris, M.E., 2012. Pharmacokinetics, lymph node uptake, and mechanistic PK model of near-infrared dye-labeled bevacizumab after IV and SC administration in mice. AAPS J. 14 (2), 252–261. https://doi.org/10.1208/s12248-012-9342-9.

Zeb, A., Ali, S.R., Rohra, D.K., 2007. Mechanism underlying hypertension and proteinuria caused by bevacizumab. J. Coll. Physicians Surg. Pak. 17 (7), 448–449.