

Vitamin D and tibial bone density, geometry, and microarchitecture in male military recruits: an observational study and randomised controlled trial

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Abstract

Vitamin D may mitigate bone stress injuries in military training by modulating changes in bone. This cross-sectional observational study (Study 1) and randomised controlled trial (Study 2) investigated associations between vitamin D metabolites and tibial structure and density, and the effect of vitamin D supplementation on tibial adaptations to military training. A total of 343 (Study 1) and 194 (Study 2) male British Army recruits participated. Circulating vitamin D metabolites (biologically ‘active’ and ‘inactive’) and tibial structure were measured in participants during week 1 (Study 1 only) and week 12 of initial military training. Associations between vitamin D metabolites and HRpQCT outcomes at week 1 were tested in Study 1. Participants in Study 2 were randomly assigned to vitamin D (oral pill or simulated sunlight) or placebo (placebo pill or placebo simulated sunlight) supplementation for 12 weeks designed to achieve vitamin D sufficiency. There was no association between total 25(OH)D or vitamin D receptor single-nucleotide polymorphisms and any measure of density, geometry, or microarchitecture ($p \geq 0.063$). Higher 1,25(OH)₂D was associated with lower cortical porosity and perimeter ($p \leq 0.040$). Higher total 24,25(OH)₂D was associated with higher trabecular number and lower trabecular thickness ($p = 0.016$). Higher 25(OH)D:24,25(OH)₂D (VMR 1) was associated with higher trabecular thickness, trabecular separation, and cortical porosity ($p \leq 0.034$). Higher 1,25(OH)₂D:24,25(OH)₂D (VMR 2) was associated with lower trabecular number, and higher trabecular spacing and thickness ($p \leq 0.035$). There was no effect of vitamin D supplementation on any tibial outcome. Training decreased trabecular area (−0.1%), thickness (−4.4%), and separation (−2.1%), and increased cortical thickness (0.8%) and area (0.9%) ($p \leq 0.042$). Vitamin D metabolites and their ratios were associated with tibial size and microarchitecture, but vitamin D supplementation had no impact on the adaptive response to military training.

Key Words: Military; Musculoskeletal Injury; Nutrition; Sunlight Exposure; Stress Fracture.

26 **Lay Summary**

27 The concentrations of active and inactive metabolites of vitamin D in blood, but not vitamin D
28 status, were associated with tibial bone structure in male military recruits. Vitamin D
29 supplementation, by oral pill or simulated sunlight, was successful increasing circulating
30 vitamin D levels in male military recruits during training in winter. Military training resulted
31 in an increase in cortical bone size, however, vitamin D supplementation had no effect on the
32 tibial bone adaptive response to military training.

Introduction

Vitamin D is hydroxylated to 25-hydroxyvitamin D (25(OH)D) in the liver before hydroxylation to either 1,25-dihydroxyvitamin D (1,25(OH)₂D) in the kidney or 24,25-dihydroxyvitamin D (24,25(OH)₂D).^(1–5) The metabolite 1,25(OH)₂D is responsible for most of the biological actions of vitamin D, but 24,25(OH)₂D—the preferred metabolite in catabolism of 25(OH)D—has its own biological actions.⁽²⁾ 1,25(OH)₂D increases intestinal absorption of calcium and phosphate to maintain mineral homeostasis, but can also act directly on bone through the vitamin D receptor.^(2–4) The role of 24,25(OH)₂D in human bone is not clear, but animal data suggest this metabolite can increase calcium and phosphate levels and promote mineralisation⁽⁶⁾ and act as a surrogate marker of vitamin D receptor activity, particularly when expressed relative to 25(OH)D.⁽⁷⁾ Several single-nucleotide polymorphisms (SNPs) in the vitamin D receptor gene may also contribute to bone outcomes;^(8–10) SNPs are genetic variants at a single base position in the gene that may change the level of gene expression or the protein sequence and expression. The vitamin D pathway is complex: parathyroid hormone (PTH)—which has its own catabolic and anabolic actions on bone—stimulates the production of, and is inhibited by, 1,25(OH)₂D. The metabolite 1,25(OH)₂D can also promote its own catabolism and inhibit its own production, stimulate bone formation, and stimulate and inhibit bone resorption.^(2–4,11,12)

Military training is characterised by a sudden increase in unaccustomed, high-impact, and repetitive lower limb mechanical loading, which can lead to bone stress injuries through targeted remodelling of fatigue damage.⁽¹³⁾ Several studies have shown changes in tibial geometry and density measured by peripheral quantitative computed tomography (pQCT) or high-resolution pQCT (HRpQCT) following 8 to 14 weeks military training.^(14–20) These studies provide evidence of adaptive bone formation, and strategies that promote bone

formation may reduce bone stress injury risk. The prevalence of vitamin D deficiency and insufficiency (serum total 25(OH)D <30 and 30 to 50 nmol·L⁻¹) in British Army recruits in winter is 31% and 41%.⁽²¹⁾ Higher total 25(OH)D⁽²²⁾ and a greater conversion of 25(OH)D to 24,25(OH)₂D⁽²¹⁾ have been associated with a lower incidence of bone stress injury in military training, indicating that vitamin D metabolites and their ratios may be important in bone health. Additionally, the vitamin D receptor rs2228570 polymorphism has been associated with bone stress injury risk and calcaneal stiffness⁽²³⁾ and bone turnover⁽²⁴⁾ in military personnel. The association between vitamin D metabolites and vitamin D receptor SNPs and tibial bone structure is poorly understood. Vitamin D deficiency has been associated with larger declines in tibial cortical volumetric bone mineral density (vBMD) during 8 weeks of military training.⁽¹⁴⁾ A 1000 international units per day (IU·d⁻¹) vitamin D and 2000 mg·d⁻¹ calcium supplement promoted increases in tibial cortical vBMD and cortical thickness during 9 weeks of basic military training.⁽²⁵⁾ Subsequent studies have shown that vitamin D (1000 IU·d⁻¹) and calcium supplementation (1000 or 2000 mg·d⁻¹) did not affect tibial bone adaptation to 8 or 12 weeks of military training,^(26,27) however, these studies were largely conducted in summer when 25(OH)D is highest.⁽²¹⁾ Only one published study has examined the effects of vitamin D supplementation on HRpQCT outcomes during military training, but this study employed a training course of only 8 weeks in duration and supplementation started in spring or summer when few participants (<20%) were vitamin D insufficient or deficient (total 25(OH)D <50 nmol·L⁻¹).⁽²⁷⁾

Understanding the associations between vitamin D metabolites and tibial bone structure, and the effect of vitamin D supplementation on the adaptive bone response to military training, may inform strategies for reducing bone stress injury risk. Vitamin D deficiency⁽²¹⁾ and tibial bone stress injuries⁽²⁸⁾ are highly prevalent in the physically arduous British Army infantry training.

This manuscript presents an observational study exploring associations between vitamin D metabolites, vitamin D receptor SNPs, and tibial bone structure (Study 1), and a randomised controlled trial investigating the effect of vitamin D supplementation on tibial adaptive bone formation (Study 2). We hypothesised that higher circulating total 25(OH)D and 1,25(OH)₂D are positively associated with tibial density, size, and microarchitecture, and vitamin D supplementation will promote adaptation in tibial bone.

Materials and Methods

This manuscript presents findings from two separate studies. Study 1 presents bone outcomes from a larger observational cross-sectional study exploring vitamin D status and illness, infection, and performance outcomes,^(21,29–33) and Study 2 presents bone outcomes from a larger randomised controlled trial exploring the effect of vitamin D supplementation on illness, infection, and physical performance outcomes^(30–32) in military recruits.

Participants

All participants were male British Army infantry trainees recruited during week one of their infantry basic military training course at the Infantry Training Centre, Catterick, UK (latitude 54°N). Women were excluded from infantry roles at the time of data collection. Participants had passed their medical assessment and were declared free of any injury or health condition precluding military training. Each participant had the study procedures and risks fully explained verbally and in writing. Written informed consent was obtained from all participants. All procedures were approved by the Ministry of Defence Research Ethics Committee (refs:165/Gen/10 and 692/MoDREC/15). For Study 1, the study was advertised from January 2014 to July 2017. For Study 2, the study was advertised during January and February 2016 and 2017 when ambient ultraviolet B was negligible in the UK; participants were eligible if

they had sun-reactive skin type I to IV, were not currently consuming vitamin D supplements, and had not used a sun bed or travelled to a sunny climate in the three months before the study.

Study Design: Study 1

This study was a cross-sectional study exploring relationships between vitamin D metabolites and tibial density, geometry, and microarchitecture. All data were collected at the start (week 1) of basic military training before any physical activity. Venous blood samples were drawn for analysis of vitamin D metabolites. Tibial structure was measured at the ultra-distal tibia by HRpQCT. Body mass, height, and body composition by DXA were measured. Childhood exercise volume and smoking status were measured by questionnaire.

Study Design: Study 2

This study was a randomised double-blind placebo-controlled trial that explored the effect of vitamin D supplementation on bone metabolism and tibial density, geometry, and microarchitecture adaptations to basic military training. All participants were completing the 26-week British Army infantry basic training course, divided into two phases (Phase One: 14 weeks basic training; Phase Two: 12 weeks specialist infantry training). The first 14 weeks of British Army infantry basic military training prepares civilians for military service and involves periods of aerobic endurance training, strength and conditioning, military specific fitness training (obstacle course, circuit training), military drill, progressive loaded marching, and basic military skills (field exercise, weapon handling). The last two weeks of infantry training involve a decrease in military activities and an increase in administrative tasks in preparation for completing training. British Army recruits complete high volumes of weight-bearing exercise and have high exercise energy expenditures (typically $> 4,000 \text{ kcal} \cdot \text{d}^{-1}$ for men),^(34,35) and we have previously shown that British Army infantry training results in marked increases

in tibial bone density and size.⁽¹⁶⁾ Participants were block randomised (parallel 1:1) to one of four 12-week interventions: i) solar-simulated ultraviolet radiation; ii) solar-simulated ultraviolet radiation placebo; iii) oral vitamin D₃; or iv) oral placebo. The solar-simulated radiation and oral vitamin D₃ were designed to restore, and then maintain, vitamin D sufficiency. Participants completed a four-week restoration phase followed by an eight-week maintenance phase. Solar-simulated ultraviolet radiation was provided as described previously to achieve 25(OH)D $\geq$ 50 nmol·L⁻¹ in most white skinned individuals.⁽³²⁾ Participants were exposed three times per week during the restoration phase and once per week during the maintenance phase to a constant ultraviolet radiation dose using a whole-body irradiation cabinet (Hapro Jade, Kapelle, Netherlands) fitted with Arimed B fluorescent tubes (Cosemidico, Stuttgart, Germany), which emitted an ultraviolet radiation spectrum similar to sunlight (λ , 290–400 nm, 95% ultraviolet A, 320–400 nm, 5% ultraviolet B, 290–320 nm). During each exposure, participants received a 1.3 $\times$ standard erythemal dose, wearing shorts and T-shirt. For the placebo, participants had the same number and duration of exposures, but the fluorescent tubes were covered with transparent ultraviolet radiation blocking film (DermaGard ultraviolet film; SunGard, Woburn, MA). Participants receiving the oral vitamin D₃ consumed a daily vitamin D₃ capsule containing 1000 IU during the restoration phase and 400 IU during the maintenance phase (Pure Encapsulations, Sudbury, MA). The restoration dose was based on previous modelling and extensive piloting to achieve 25(OH)D $\geq$ 50 nmol·L⁻¹. The placebo group consumed a daily identical looking placebo capsule (Almac Group, County Armagh, UK). The vitamin D₃ content of the 1000 IU, 400 IU, and placebo capsules was 1090 IU, 460 IU, and 0 IU. Before and after the 12-week intervention tibial density, geometry, and microarchitecture were measured by HRpQCT, and areal bone mineral density (aBMD) was measured by DXA. Venous blood samples were taken at weeks 1, 5, and 12 to determine vitamin D status and metabolites and circulating markers of bone metabolism. The

vitamin D status data have been previously published⁽³²⁾ and show that both the solar-simulated radiation and oral vitamin D₃ treatments increased serum total 25(OH)D and 1,25(OH)₂D to a similar extent. Therefore, the two treatment groups were collapsed into one vitamin D group (solar-simulated radiation and oral vitamin D₃) and the two placebo groups were collapsed into one placebo group (solar-simulated radiation placebo and oral placebo) resulting in one intervention group and one control group.

Tibial Volumetric Bone Mineral Density, Geometry, and Microarchitecture

First-generation HRpQCT (XtremeCT, Scanco Medical AG, Switzerland) was used to assess vBMD, geometry, and microarchitecture of the ultra-distal tibia in the non-dominant leg (Study 1) and right leg (Study 2). Leg dominance was self-determined as the leg most likely used to kick a ball. The right leg was chosen for Study 2 as the tibial adaptive response to military training is similar between dominant and non-dominant legs.⁽¹⁶⁾ A 3D representation of 9.02 mm of the tibia in the axial direction was obtained from 110 slices with an isotropic voxel size of 82 µm. The leg was fitted into a carbon fibre shell and immobilised within the scanner gantry. A reference line was positioned at the tibial endplate with the first slice taken from 22.5 mm proximal to the reference line. Daily quality control scans were performed using the manufacturer issued phantom. The quality of each scan was reviewed by a single operator according to manufacturer instructions and any poor-quality scans were repeated. The manufacturer's standard evaluation procedure was used to derive: total, trabecular, and cortical vBMD (mg HA·cm⁻³); trabecular area (mm²); trabecular bone volume fraction (%); trabecular number (1·mm⁻¹); trabecular thickness (µm); trabecular separation (µm); cortical area (mm²); cortical thickness (mm), and; cortical perimeter (mm). A semi-automated segmentation technique was used to determine cortical porosity (%). Automated 2D registration was used for longitudinal analysis in Study 2 and participants with <75% common region were excluded.⁽³⁶⁾

Micro-finite element analysis was performed using the manufacturer's software to estimate stiffness ($\text{kN}\cdot\text{mm}^{-1}$) and failure load (kN). All scans and evaluations were performed by a single investigator.

Blood Collection

A venous blood sample was collected either in the morning (0900 to 1100 h) or early afternoon (1300 to 1500 h). It was not possible to standardise the blood sample for the same time of day due to the nature of military training, but time of day was matched between week 1 and week 12 in Study 2 to ensure circadian patterns were controlled for. Venous blood was drawn from a vein in the antecubital fossa and collected in serum and EDTA tubes (Becton Dickinson, New Jersey, USA). Serum samples were left to clot for 1 hour at room temperature. Blood samples were centrifuged at 1500 g and 4°C for 10 min before serum and plasma were separated and stored at -80°C until the time of analyses.

Biochemical Analyses

Plasma procollagen type I N-terminal propeptide (PINP), c-telopeptide cross-links of type I collagen (βCTX), and PTH were analysed by electro-chemiluminescence immunoassays on the COBAS c601 platform (Roche Diagnostics, Mannheim, Germany). Serum samples were analysed for total 25(OH)D (sum of 25(OH)D₂ and 25(OH)D₃) and total 24,25(OH)₂D (sum of 24,25(OH)₂D₂ and 24,25(OH)₂D₃) by high-performance liquid chromatography tandem mass spectrometry using a Micromass Quattro Ultima Pt electrospray ionisation mass spectrometer. The 25(OH)D₃ and 25(OH)D₂ assays were calibrated using the National Institute of Science and Technology standard reference material SRM972a. Serum 1,25(OH)₂D was measured by chemiluminescent immunoassay using a DiaSorin LIAISON® XL analyser (Stillwater, Minnesota, USA). The vitamin D metabolite ratios (VMR) 25(OH)D:24,25(OH)₂D (VMR 1)

and 1,25(OH)₂D:24,25(OH)₂D (VMR 2) were calculated.^(21,29,33,37) All analyses were undertaken according to Good Clinical Laboratory Practice and by the Vitamin D External Quality Assessment Scheme certified Bioanalytical Facility, University of East Anglia, Norwich, UK. Assay precisions are presented in Supplementary Material 1.

Single-Nucleotide Polymorphisms

Whole blood samples in EDTA vacutainers were defrosted and resuspended for 15 min on a rotating wheel. Genomic DNA was isolated from whole blood using the ReliaPrep™ Blood gDNA Miniprep System (Promega, Southampton, UK) according to manufacturer's instructions. Using samples of DNA, Kompetitive Allele Specific PCR (KASP™, LGC Genomics, Teddington, Middlesex, UK) genotyping was used for SNP genotyping of rs2228570, rs4516035, and rs7139166 in the vitamin D receptor gene. All three polymorphisms have been associated with total 25(OH)D⁽³⁸⁾ with the rs2228570 polymorphism previously associated with bone stress injury and lower limb bone quality outcomes in male military personnel,⁽²³⁾ and the rs4516035 polymorphism previously associated with osteoporosis.⁽³⁹⁾ Genotypes were designated as TT, CC, or CT alleles for the rs2228570 and rs4516035 SNPs, and GG, CC, or CG alleles for the rs7139166 SNP.

Whole-Body Areal Bone Mineral Density

Whole-body lean mass, fat mass, and aBMD were assessed by DXA (Lunar iDXA, GE Healthcare, Buckinghamshire, UK), with participants wearing underwear. The CV for whole-body aBMD, lean mass, and fat mass is 0.5%, 0.5%, and 1.1%. Regional aBMD was derived from the whole-body scan.

Statistical Analyses: Study 1

All data were analysed using the R programming language (v.4.4.1). These were secondary analyses^(21,29–31,33) and so no *a priori* sample size was calculated. Generalized additive models (*mgcv* package v.1.9-1) were used to test associations between each vitamin D metabolite or their ratios (total 25(OH)D, 1,25(OH)₂D, total 24,25(OH)₂D, VMR 1, and VMR 2) and tibial structure (HRpQCT outcomes) controlling for age, height, lean mass, fat mass, childhood exercise volume, and smoking status.⁽³³⁾ Smoothing splines were assigned to each continuous variable with the knots unspecified and the model fitted with restricted maximum likelihood estimation. Variance and normality of the residuals were checked visually, and k values were checked against $p \leq 0.05$. Only one model revealed non-normal residuals; the residuals in the VMR 1 models revealed right skew. One participant had very high VMR 1 (approximately nine standard deviations above the mean) and was removed from analysis. The significance of the smooth terms was accepted as $p \leq 0.05$. The deviance explained, which is interpreted like R^2 , for the individual vitamin D metabolites was determined by comparing the deviance explained for the generalised model to a null model (the same generalized additive model without the vitamin D metabolite included). A one-way ANCOVA (*car* package v.3.1-3) was used to compare HRpQCT outcomes between individuals grouped based on alleles of the rs2228570 (TT vs CC vs CT), rs4516035 (TT vs CC vs CT), and rs7139166 (GG vs CC vs CG) polymorphisms, controlling for age, height, lean mass, fat mass, childhood exercise volume, and smoking status.⁽³³⁾ Significance accepted as $p \leq 0.05$.

Statistical Analyses: Study 2

These were secondary analyses of a randomised controlled trial^(30–32) and so a sensitivity analysis rather than an *a priori* sample size was calculated. Sensitivity analysis revealed that our sample size (based on a two-group trial with a minimum of 33 per group), was adequate to detect a small to medium effect size ($f^2 \geq 0.176$) with an alpha of 0.05 and a power of 80% (*pwr*

package v.1.3-0). Linear mixed effect models with restricted maximum likelihood estimation were used to examine changes in HRpQCT, DXA, and blood biomarker outcomes between vitamin D and placebo groups. Group (vitamin D vs placebo), time (week 1 vs week 5 [blood biomarkers only] vs week 12), and their interaction were included as fixed effects. Random intercepts were assigned to each participant to account for within participant correlation for repeated measures. The significance of the fixed effects was determined with Satterthwaite degrees of freedom (*lmerTest* package v.3.1.3). In the event of a significant interaction, pairwise uncorrected comparisons were used to identify differences between time points or groups (*emmeans* package v.1.10.4). Pooled data were used for main effects when there was no significant interaction, and each group was analysed independently when there was a significant interaction. Normality of the residuals were checked visually by plotting the residuals against the fitted values and from Q-Q plots. The residuals for total and arms aBMD, total and trabecular vBMD, trabecular volume and spacing, and cortical porosity and perimeter had long-tailed distributions, but no model had skewed distribution, histograms of original data revealed no obvious influential data points, and this study had a reasonable sample size, and so the models were interpreted under the assumption of normality. The residuals for VMR 1 and VMR 2 also had long-tailed distributions, which were successfully corrected by log-transformation. Effect sizes are presented as partial eta-squared (η_p^2) for main and interaction effects, Hedges' *g* for between-group comparisons, and paired Hedges' *g* for within-group paired comparisons (*effectsize* package v.0.6.0.1). Figures were drawn in the *ggplot2* package (v.3.4.2). Significance was accepted as $p \leq 0.05$.

Results

Study 1: Participants

A total of 1332 male infantry recruits volunteered. A random convenience sample of 343 participants were selected for HRpQCT measurements (Table 1). Only 343 participants could be included in the HRpQCT outcomes due to time constraints in the military training programme. Participants were recruited throughout the year (spring n = 59, summer n = 104, autumn n = 123, winter n = 57).

Study 1: Associations Between Vitamin D Metabolites and Tibial Structure

There was no association between total 25(OH)D and any measure of tibial trabecular or cortical vBMD, geometry, or microarchitecture ($p = 0.063$ to 0.919) (Figure 1). Higher 1,25(OH)₂D was associated with lower cortical porosity ($p = 0.005$) and lower cortical perimeter ($p = 0.040$) in an approximately linear relationship, but 1,25(OH)₂D was not associated with any other bone outcome ($p = 0.054$ to 0.777) (Figure 2). Higher total 24,25(OH)₂D was associated with higher trabecular number ($p = 0.016$) and lower trabecular thickness ($p = 0.016$) in an approximately linear relationship, but total 24,25(OH)₂D was not associated with any other bone outcome ($p = 0.052$ to 0.897) (Figure 3).

Higher VMR 1 was associated with greater trabecular thickness ($p = 0.006$) and greater trabecular separation ($p = 0.034$) until approximately 15 (arbitrary unit, range 5 to 59) (Supplementary Figure 1). Higher VMR 1 beyond 25 was also associated with higher trabecular thickness and trabecular separation, but the confidence intervals were large. Higher VMR 1 was associated with higher cortical porosity ($p = 0.009$), in an approximately linear relationship, but VMR 1 was not associated with any other bone outcome ($p = 0.078$ to 0.880). Higher VMR 2 was associated with lower trabecular number ($p = 0.018$) and greater trabecular spacing ($p = 0.027$) in an approximately linear relationship (Supplementary Figure 2). Higher VMR 2 was also associated with higher trabecular thickness until approximately 125 VMR 1

($p = 0.035$). There were no other associations between VMR 1 and bone outcomes ($p = 0.207$ to 0.867).

Study 1: Associations Between Single-Nucleotide Polymorphisms and Tibial Structure

For the rs2228570 SNP, the proportion of people with the TT, CC, and CT alleles were 17%, 36%, and 47%; for the rs4516035 SNP, the proportion of people with the TT, CC, and CT alleles were 37%, 16%, and 46%; for the rs7139166 SNP, the proportion of people with the GG, CC, and CG alleles were 46%, 38%, and 16%. There were no associations between any vitamin D receptor SNPs and bone outcomes (data not shown, $p = 0.103$ to 0.888).

Study 2: Participants

A total of 319 male infantry recruits volunteered; 29 did not meet the inclusion criteria (sunbed use $n = 13$, vitamin D supplements $n = 2$, recent holiday $n = 9$, skin type V/VI $n = 5$) and 59 withdrew before randomisation. A total of 231 completed baseline measures and 194 completed at least one follow-up measure and were included in analyses (Figure 4, Table 2). A total of 56 of these 231 had at least one musculoskeletal injury, four of which were a bone stress injury. Of these, 78 had baseline and follow-up HRpQCT scans; five participants had common regions $<75\%$ resulting in 73 participants with completed scans (median common region 95% [92%, 98%], median number of slices analysed = 105 slices [101, 108]). Daily sunlight exposure and vitamin D intake have been reported previously and were not different between groups.⁽³²⁾ All participants had more than 80% compliance to treatment and were adequately blinded to the intervention with 35% correctly guessing their allocation, 32% were incorrect, and 33% did not know.⁽³²⁾

Study 2: Vitamin D Supplementation and Vitamin D Metabolites

Circulating vitamin D metabolites and markers of bone metabolism are shown in Figure 5 with vitamin D metabolite ratios presented in Supplementary Material 2 and shown in Supplementary Figure 3. There were group $\times$ time interactions for total 25(OH)D, 1,25(OH)₂D, and 24,25(OH)₂D (all $p \leq 0.001$, $\eta_p^2 = 0.038$ to 0.431). Total 25(OH)D increased from week 1 to week 5 and week 12 (both $p < 0.001$, $g = 1.72$ to 1.73) in the vitamin D group with week 5 and week 12 not different ($p = 0.194$, $g = 0.19$). Total 25(OH)D did not change from week 1 to week 5 in the placebo group ($p = 0.709$, $g = 0.12$) but increased from week 5 to week 12 ($p < 0.001$, $g = 1.29$) with week 12 also higher than week 1 ($p < 0.001$, $g = 1.07$). Total 25(OH)D was higher in the vitamin D than placebo group at week 5 and week 12 (both $p < 0.001$, $g = 0.94$ to 2.20). The number of vitamin D sufficient participants was 95% and 94% at week 5 and week 12 for the vitamin D group and 22% and 66% at week 5 and week 12 for the placebo group. 1,25(OH)₂D increased from week 1 to week 5 and week 12 in the vitamin D group (both $p < 0.001$, $g = 0.33$ to 0.40) with week 5 and week 12 not different ($p = 0.972$, $g < 0.01$). 1,25(OH)₂D did not change from week 1 to week 5 in the placebo group ($p = 0.278$, $g = 0.15$) but increased from week 5 to week 12 ($p = 0.004$, $g = 0.35$) with week 12 not different to week 1 ($p = 0.152$, $g = 0.23$). 1,25(OH)₂D was higher in the vitamin D than placebo group at week 5 ($p = 0.002$, $g = 0.44$) but not at week 12 ($p = 0.434$, $g = 0.12$). 24,25(OH)₂D increased from week 1 to week 5 and week 12 in the vitamin D group (both $p < 0.001$, $g = 1.53$ to 1.64) with week 5 and week 12 not different ($p = 0.194$, $g = 0.17$). 24,25(OH)₂D decreased from week 1 to week 5 in the placebo group ($p = 0.017$, $g = 0.37$) and increased from week 5 to week 12 ($p < 0.001$, $g = 1.04$) with week 12 higher than week 1 ($p < 0.001$, $g = 0.41$). 24,25(OH)₂D was higher in the vitamin D than placebo group at week 5 and week 12 (both $p < 0.001$, $g = 1.24$ to 1.75).

Study 2: Vitamin D Supplementation and Bone Metabolism

Circulating markers of bone metabolism are shown in Figure 5. There was a group $\times$ time interaction for PTH ($p < 0.001$, $\eta_p^2 = 0.051$). PTH decreased from week 1 to week 5 and week 12 in the vitamin D group ($p < 0.001$ to 0.030 , $g = 0.28$ to 0.77) with week 12 lower than week 5 ($p < 0.001$, $g = 0.49$). PTH increased from week 1 to week 5 in the placebo group ($p = 0.040$, $g = 0.21$) and decreased from week 5 to week 12 ($p = 0.015$, $g = 0.27$) with week 1 and week 12 not different ($p < 0.837$, $g = 0.07$). PTH was lower in the vitamin D than placebo group at week 5 and week 12 ($p = 0.003$ to 0.010 , $g = 0.35$ to 0.60). There was no effect of vitamin D supplementation (main effects of group, $p = 0.258$ to 0.943 , $\eta_p^2 = 0.007$ to 0.021 ; group $\times$ time interactions, both $p = 0.716$ to 0.753 , both $\eta_p^2 = 0.002$) on β CTX and PINP but there were main effects of time (both $p \leq 0.001$, $\eta_p^2 = 0.249$ to 0.265). β CTX decreased from week 1 to week 5 and week 12 when the level of group was collapsed (both $p < 0.001$, $g = 0.68$ to 0.72) with week 5 and week 12 not different ($p = 0.987$, $g = 0.02$). PINP increased from week 1 to week 5 and week 12 when the level of group was collapsed ($p < 0.001$ to 0.048 , $g = 0.13$ to 0.76) and decreased from week 5 to week 12 ($p < 0.001$, $g = 0.67$).

Study 2: Vitamin D Supplementation, Areal Bone Mineral Density, and Tibial Structure

Total body and regional aBMD are shown in Supplementary Figure 4. There was no effect of vitamin D supplementation on aBMD at any site (main effects of group, all $p = 0.305$ to 0.811 , $\eta_p^2 = 0.001$ to 0.010 ; group $\times$ time interactions, all $p = 0.318$ to 0.705 , $\eta_p^2 = 0.002$ to 0.012). Training increased legs aBMD ($0.9 \pm 1.9\%$, main effect of time, $p < 0.001$, $\eta_p^2 = 0.173$) but had no effect on total, arms, or trunk aBMD when collapsing the level of group (main effects of time, all $p = 0.187$ to 0.366 , $\eta_p^2 = 0.010$ to 0.021).

Tibial density, geometry, and microarchitecture are shown in Table 3. There was no effect of vitamin D supplementation (main effects of group, all $p = 0.146$ to 0.958 , $\eta_p^2 < 0.001$ to 0.030 ;

group $\times$ time interactions, all $p = 0.186$ to 0.900 , $\eta_p^2 < 0.001$ to 0.030) or training (main effects of time, all $p = 0.097$ to 0.911 , $\eta_p^2 < 0.001$ to 0.038) on total vBMD, trabecular vBMD, trabecular volume, trabecular number, cortical vBMD, cortical porosity, stiffness, or failure load. There was no effect of vitamin D supplementation (main effects of group, all $p = 0.080$ to 0.942 , $\eta_p^2 < 0.001$ to 0.043 ; group $\times$ time interactions, all $p = 0.071$ to 0.461 , $\eta_p^2 = 0.008$ to 0.045) but training decreased trabecular area ($-0.1 \pm 0.5\%$), trabecular thickness ($-2.1 \pm 10.1\%$), trabecular separation ($-4.4 \pm 18.4\%$), and increased cortical area ($0.9 \pm 2.6\%$) and cortical thickness ($0.8 \pm 2.9\%$) (main effects of time, all $p = 0.008$ to 0.042 , $\eta_p^2 = 0.057$ to 0.096).

Discussion

Vitamin D metabolites and their ratios were associated with tibial size and microarchitecture in male military recruits. Military training resulted in adaptations to cortical geometry and trabecular microarchitecture at the ultra-distal tibia, but vitamin D supplementation had no impact on the adaptive response to training. Only one study has used HRpQCT to examine the effects of vitamin D supplementation on tibial bone adaptation to military training, showing no effect of supplementation over 8 weeks of training in summer (when few participants [$<20\%$] were vitamin D deficient or insufficient [$25(\text{OH})\text{D} < 50 \text{ nmol}\cdot\text{L}^{-1}$]).⁽²⁷⁾ Our data show vitamin D supplementation in winter—where over 75% of participants were vitamin D deficient or insufficient and supplementation restored 95% of participants to vitamin D sufficiency—had no effect on ultra-distal tibial adaptation to a longer period of military training. The participants in this study were completing infantry training, one of the British Army's most arduous courses with a high risk of bone stress injuries.⁽²⁸⁾

Vitamin D Metabolites and Tibial Structure

407 Total 25(OH)D was not associated with any measure of tibial density, geometry, or
 408 microarchitecture, but higher 1,25(OH)₂D was associated with lower cortical porosity and
 409 cortical perimeter, although the amount of variance explained was small for all metabolites.
 410 1,25(OH)₂D has a short half-life and is tightly regulated, demonstrating no relationship with
 411 25(OH)D.⁽³⁷⁾ Data from other HRpQCT observational studies show positive associations
 412 between 25(OH)D and tibia trabecular vBMD, cortical area, and/or trabecular
 413 microarchitecture in young,⁽⁴⁰⁾ middle aged,⁽⁴¹⁾ and older^(42,43) women and/or men; the
 414 homogeneity of our population (*i.e.*, all had passed military fitness standards) may explain why
 415 we did not observe associations between total 25(OH)D and bone outcomes. There are limited
 416 vitamin D metabolite and HRpQCT data previously published. Higher 1,25(OH)₂D could
 417 lower cortical porosity through increased mineralisation from increased calcium and phosphate
 418 availability, and by inhibiting PTH-induced bone resorption in cortical bone.^(4,12) It is not clear
 419 why higher 1,25(OH)₂D was also associated with smaller cortical perimeter, but vitamin D
 420 supplementation has been shown to decrease cortical perimeter during military training⁽²⁵⁾
 421 possibly through an increase in remodelling; higher 1,25(OH)₂D can increase bone resorption
 422 on the periosteal surface through stimulating osteoblast receptor activator of nuclear factor
 423 kappa B ligand (RANKL) production.⁽⁴⁾ High dose vitamin D supplementation (1800 to 10,000
 424 IU·d⁻¹) has also shown to deteriorate aspects of tibial trabecular microarchitecture and/or
 425 cortical vBMD in fracture patients,⁽⁴⁴⁾ postmenopausal women with secondary
 426 hyperparathyroidism,⁽⁴⁵⁾ and older adults;⁽⁴⁶⁾ however, the deterioration appears associated
 427 with the increase in 24,25(OH)₂D rather than 1,25(OH)₂D.⁽⁴⁷⁾ In our study, higher total
 428 24,25(OH)₂D was associated with higher trabecular number and lower trabecular thickness
 429 providing new evidence that different vitamin D metabolites have different associations with
 430 cortical and trabecular bone. The role of 24,25(OH)₂D in human bone is not clear, but animal

data suggest this metabolite can increase calcium and phosphate levels and promote mineralisation.⁽⁶⁾

Higher VMR 1 was associated with greater trabecular thickness, trabecular separation, and cortical porosity. Conversion of 25(OH)D to 24,25(OH)₂D is stimulated by 1,25(OH)₂D to prevent tissue level toxicity^(5,7) and so 24,25(OH)₂D and the ratio between 24,25(OH)₂D and 25(OH)D could be surrogate markers of vitamin D receptor activity.⁽⁷⁾ Higher production of 24,25(OH)₂D relative to 25(OH)D (*i.e.*, higher 24,25(OH)₂D:25(OH)D) was associated with higher tibial total and trabecular vBMD in older men,⁽⁷⁾ consistent with our finding of lower trabecular separation with lower VMR 1, but it is unclear why trabecular thickness was also greater with higher VMR 1. The rs2228570 polymorphism has been associated with bone stress injury risk and calcaneal stiffness index measured by ultrasound,⁽²³⁾ and lower values of biochemical markers of bone turnover⁽²⁴⁾ in military personnel, but we did not observe any association between vitamin D receptor polymorphisms and HRpQCT outcomes with the few SNPs we selected. Higher VMR 2 was associated with lower trabecular number and higher trabecular spacing and thickness. Lower VMR 2 and higher 24,25(OH)₂D⁽²¹⁾ have been associated with a lower incidence of bone stress injury in military training, and the higher trabecular number with lower VMR 2 may contribute to this reduced risk. The associations in this study must be interpreted with caution as the effects were small and were influenced by individuals with high metabolite values.

Vitamin D Supplementation and Tibial Structure

Vitamin D supplementation increased total 25(OH)D and 1,25(OH)₂D compared with placebo. Total 25(OH)D and 1,25(OH)₂D increased in the placebo group by week 12, likely due to increased sun exposure^(16,17,27) and release from fat stores.⁽¹⁴⁾ Circulating 24,25(OH)₂D showed

a similar pattern, consistent with increased catabolism of 25(OH)D. Vitamin D supplementation had no effect on tibial adaptation to military training. Conversely, vitamin D (1000 IU·d⁻¹) and calcium (2000 mg·d⁻¹) supplementation augmented increases in pQCT-derived tibial cortical vBMD (4% site) and cortical thickness (14% site) following 9 weeks military training in men and women.⁽²⁵⁾ Vitamin D deficiency has also been associated with a greater decrease in tibial cortical vBMD following 8 weeks military training in women measured by HRpQCT.⁽¹⁴⁾ Cortical vBMD was unchanged in our study and the cortical vBMD response at the distal tibia to military training is inconsistent with increases,^(16,19,20) decreases,⁽¹⁴⁾ and no change^(17,18) reported.

It is possible we did not observe an effect of vitamin D supplementation on tibial outcomes because vitamin D may largely affect cortical bone, and our analysis was limited to the highly trabecular ultra-distal tibia. Military training can elicit periosteal expansion at the tibia^(15,17,18,27) with periosteal apposition an important part of the formation modelling response to increased loading.⁽⁴⁸⁾ Our HRpQCT methods may have been unable to detect an effect of vitamin D on cortical bone; periosteal expansion is difficult to detect as the 2D registration method matches baseline and follow-up scans by cross-sectional area, and the first generation HRpQCT can lack resolution in detecting smaller cortical pores in the assessment of cortical porosity.⁽³⁶⁾ Increased 1,25(OH)₂D could promote cortical mineralisation and periosteal expansion through increasing calcium and phosphate intestinal absorption or by inhibiting PTH secretion, which increases bone resorption—predominantly in cortical bone—by stimulating RANKL and inhibiting osteoprotegerin (OPG);^(3,11,12) the periosteal cells appear more sensitive to PTH than endosteal cells.⁽⁴⁹⁾ Vitamin D suppressed PTH in this study, consistent with other military training studies showing vitamin D and calcium supplementation suppressed PTH and increased OPG:RANKL,⁽²⁵⁾ yet we did not observe a difference in markers of bone metabolism

between groups. Evidence from transgenic mouse models shows 1,25(OH)₂D can also directly promote periosteal bone formation through the vitamin D receptor on osteoblasts.⁽⁵⁰⁾ Nevertheless, our findings are supported by other studies showing vitamin D (1000 IU·d⁻¹) and calcium (1000 or 2000 mg·d⁻¹) supplementation had no effect on tibial adaptations to 8 to 12 weeks military training.^(26,27)

Training increased cortical thickness (0.8%) and cortical area (0.9%), consistent with previous HRpQCT studies in military training.^(14,16,18–20) Increased cortical thickness could develop from formation modelling on either the periosteal or endosteal surface.⁽⁴⁸⁾ Training also decreased trabecular area (–0.1%), consistent with previous HRpQCT studies of basic military training^(14,16,18) and animal studies of bone formation,⁽⁵¹⁾ indicative of endocortical contraction.^(16,18) We were unable to determine if training resulted in periosteal apposition using our 2D registration method⁽³⁶⁾ but small increases in circumference add considerably to strength and fracture resistance.⁽⁵²⁾ Periosteal expansion may protect against bone stress injury risk by providing strength in bending,⁽⁵²⁾ offsetting temporary weakening due to increased porosity during remodelling of fatigue damage.⁽⁴⁸⁾ The clinical and mechanical implications of periosteal expansion at the distal tibia are unclear as the distal tibia is subject to different and complex loading patterns with most of the forces at the distal tibia compressive^(15,16) with some tensile strains,⁽⁵³⁾ and bone stress injuries do not typically develop at the ultra-distal site during basic military training. Training decreased trabecular separation (–4.4%) consistent with the formation of new bone on trabecular surfaces,⁽¹⁴⁾ however, we observed a decrease in trabecular thickness (–2.1%) and no change in trabecular number. These structural adaptations to mechanical loading did not lead to improved estimated mechanical strength (failure load) under compression and suggest cortical adaptations have minimal impact in increasing strength at the ultra-distal site. The loading also likely caused a number of bone modelling and remodeling

cellular responses that take longer than the follow-up time in this study to observe structural or mechanical effects.^(48,54) Previous military training studies show decreased trabecular separation,^(14,18–20,27) and increased^(14,18–20,27) or unchanged⁽¹⁶⁾ trabecular thickness and number. The decrease in trabecular number and thickness could be due to endocortical contraction, an increase in remodelling on the trabecular surfaces, or because adaptations to trabecular microarchitecture can take longer than 12 weeks.⁽¹⁸⁾ Differences between studies could be attributed to our use of the first-generation HRpQCT, whereas most previous studies used higher resolution second-generation HRpQCT.^(14,18–20) Although there was no interaction between the treatment group and time, the effect size for a decrease in trabecular thickness was three times as large for the vitamin D compared with the placebo group, and vitamin D supplementation may deteriorate aspects of trabecular microarchitecture due to upregulating catabolism of 1,25(OH)₂D or by stimulating RANKL production from osteoblasts.⁽⁴⁴⁾

Limitations

We did not correct our analyses for the multiple HRpQCT and metabolic outcomes, and our data should be interpreted considering the chance of type I error. Our data were generated from first-generation HRpQCT, which has a lower resolution than a second-generation scanner and cannot measure more proximal tibia sites. Our study is susceptible to survivor bias, where only those who completed 12 weeks of training were followed up; however, we have previously shown that demographic and bone outcomes from DXA and HRpQCT are not different between those who complete training and those who do not.⁽¹⁶⁾ We were unable to include a measure of physical activity or exercise completed by recruits in training, which would help explain some of the adaptive bone formation associated with novel mechanical loading. It is possible that some participants may have not reached peak bone mass in the appendicular skeleton or scans were performed in the presence of an unfused or fusing growth plate,

however, the minimum age of participants was 18 years and no unfused growth plates were observed, and age was controlled for in the models in Study 1. Our study did not control for ethnicity due to the low numbers of non-white individuals and further work is required to better understand the role of ethnicity in the vitamin D and bone relationship. Finally, our sample size was small, and there were likely effects that we could not detect, but these effects would be small.

Conclusions

Vitamin D metabolites and vitamin D metabolite ratios were associated with tibial size and microarchitecture in healthy young men; however, vitamin D supplementation had no impact on the bone adaptive response to 12 weeks military training.

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Author Contributions

JPG conceived the study. JPG, NPW, SJ, and SJO designed the study. DA and LER designed and provided dosimetry for the ultraviolet radiation and sham ultraviolet radiation exposures. TJO, SJ, NPW, ATC, SJO, RI, and JPG collected the data. JCYT and WDF analysed the biochemical samples. TJO produced the manuscript and performed the data analysis. All authors edited the manuscript and approved the final version.

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- 724

725 **Table 1.** Participant demographics.

	Mean $\pm$ SD (n = 343)
Age (years)	22 $\pm$ 3
Body Mass (kg)	75.5 $\pm$ 10.1
Height (m)	1.77 $\pm$ 0.06
Body Mass Index (kg·m ⁻²)	24.0 $\pm$ 2.7
Exercise Volume (mins·week ⁻¹)	538 $\pm$ 619
Fat Mass (kg)	14.3 $\pm$ 5.6
Lean Mass (kg)	57.3 $\pm$ 6.4
Whole-body aBMD (g·cm ⁻²)	1.23 $\pm$ 0.11
Total 25(OH)D (nmol·L ⁻¹)	60.6 $\pm$ 29.2
Vitamin D Deficient (total 25(OH)D <30 nmol·L ⁻¹) (%)	17
Vitamin D Insufficient (total 25(OH)D 30 to 49 nmol·L ⁻¹) (%)	23
1,25(OH) ₂ D (pmol·L ⁻¹)	129 $\pm$ 34
Total 24,25(OH) ₂ D (nmol·L ⁻¹)	4.94 $\pm$ 3.06
VMR 1	14.0 $\pm$ 5.0
VMR 2	39.6 $\pm$ 31.0
Total Area (mm ²)	846 $\pm$ 142
Total vBMD (mg HA·cm ⁻³)	347 $\pm$ 48
Trabecular Area (mm ²)	699 $\pm$ 145
Trabecular vBMD (mg HA·cm ⁻³)	229 $\pm$ 31
Trabecular Volume (%)	19.0 $\pm$ 2.6
Trabecular Number (1·mm ⁻¹)	2.19 $\pm$ 0.30
Trabecular Thickness (μm)	88 $\pm$ 12
Trabecular Separation (μm)	378 $\pm$ 63
Cortical Area (mm ²)	139 $\pm$ 20
Cortical vBMD (mg HA·cm ⁻³)	887 $\pm$ 38
Cortical Perimeter (mm)	114 $\pm$ 9
Cortical Porosity (%)	4.80 $\pm$ 1.61
Cortical Thickness (mm)	1.32 $\pm$ 0.24
Stiffness (kN·mm ⁻¹)	281 $\pm$ 42
Failure Load (kN)	14.08 $\pm$ 2.03

Mean ± SD
(n = 343)

1,25(OH)₂D, 1,25-dihydroxyvitamin D; aBMD, areal bone mineral density; Total 25(OH)D, total 25-hydroxyvitamin D; Total 24,25(OH)₂D, total 24,25-dihydroxyvitamin D; vBMD, volumetric bone mineral density; VMR 1, 25(OH)D:24,25(OH)₂D; VMR 2, 1,25(OH)₂D:24,25(OH)₂D.

Missing data: Total Area = 3, Total vBMD = 3, Trabecular Area = 3, Trabecular vBMD = 3, Trabecular Volume = 3, Trabecular Number = 3, Trabecular Thickness = 3, Trabecular Separation = 3, Cortical Area = 16, Cortical vBMD = 16, Cortical Perimeter = 17, Cortical Porosity = 16, Cortical Thickness = 16, Stiffness = 12, Failure Load = 12.

726

Table 2. Demographics at week 1 in the placebo and vitamin D groups. Data are mean $\pm$ SD or %.

	Placebo (n = 98)	Vitamin D (n = 96)
Age (years)	21 $\pm$ 3	21 $\pm$ 3
Body Mass (kg)	78.2 $\pm$ 10.8	76.5 $\pm$ 11.0
Body Mass Index (kg·m ⁻²)	24.7 $\pm$ 2.8	24.4 $\pm$ 3.2
Lean Mass (kg)	57.5 $\pm$ 6.2	56.0 $\pm$ 7.3
Fat Mass (kg)	17.8 $\pm$ 5.9	17.4 $\pm$ 5.6
Body Fat (%)	21.4 $\pm$ 5.7	21.6 $\pm$ 5.1
Vitamin D Status (%)		
Deficient (total 25(OH)D <30 nmol·L ⁻¹)	34	32
Insufficient (total 25(OH)D 30 to 49 nmol·L ⁻¹)	44	44

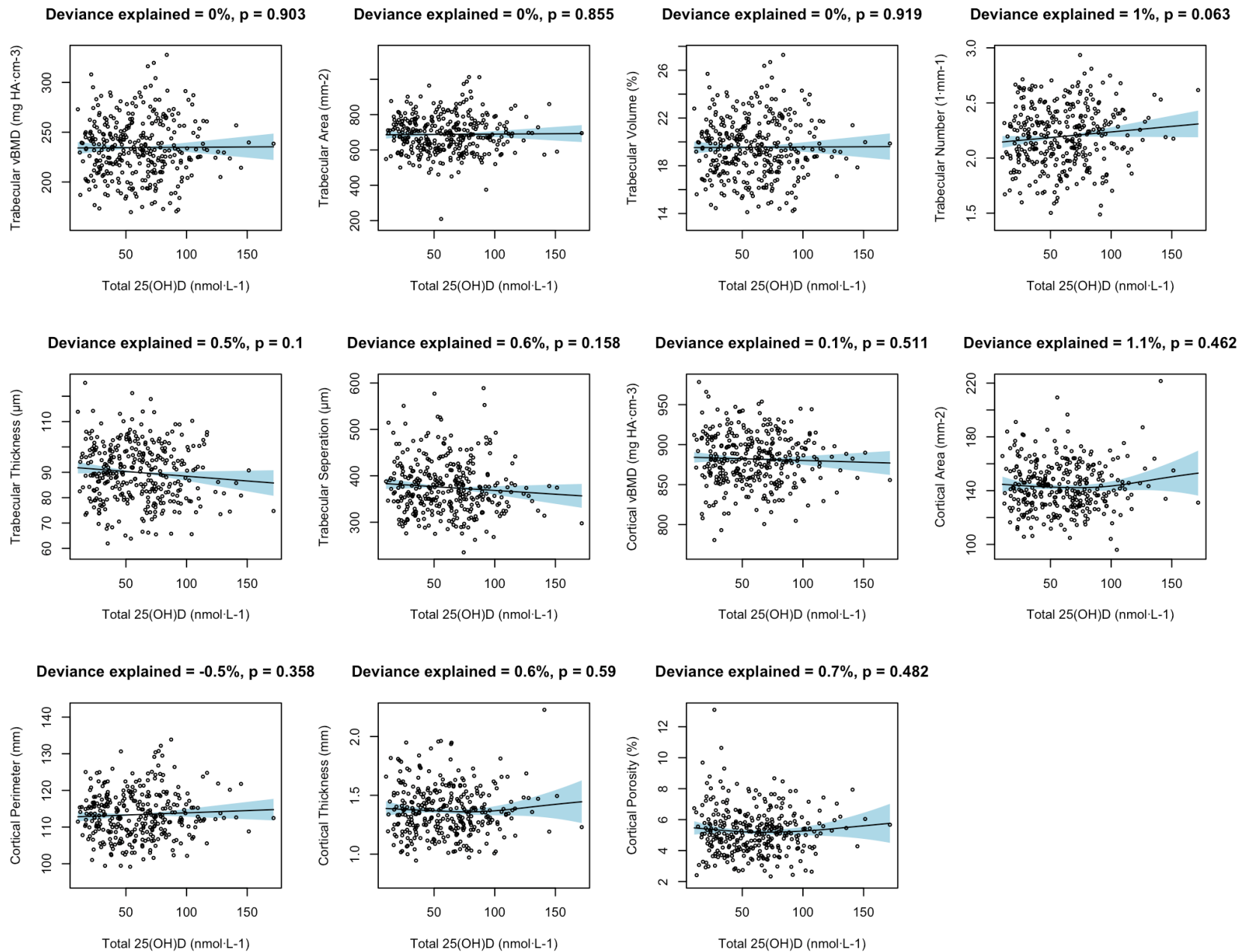
Missing data: Age = 1 Placebo; Body Mass = 1 Vitamin D, 2 Placebo; Height = 1 Vitamin D, 2 Placebo; Lean Mass, Fat Mass, and Body Fat = 44 Vitamin D, 45 Placebo.

Table 3. Changes in tibial density, geometry, and microarchitecture in response to 12 weeks basic military training in men supplemented with placebo or vitamin D. Data are mean $\pm$ SD, mean absolute unadjusted differences, and paired Hedges' g.

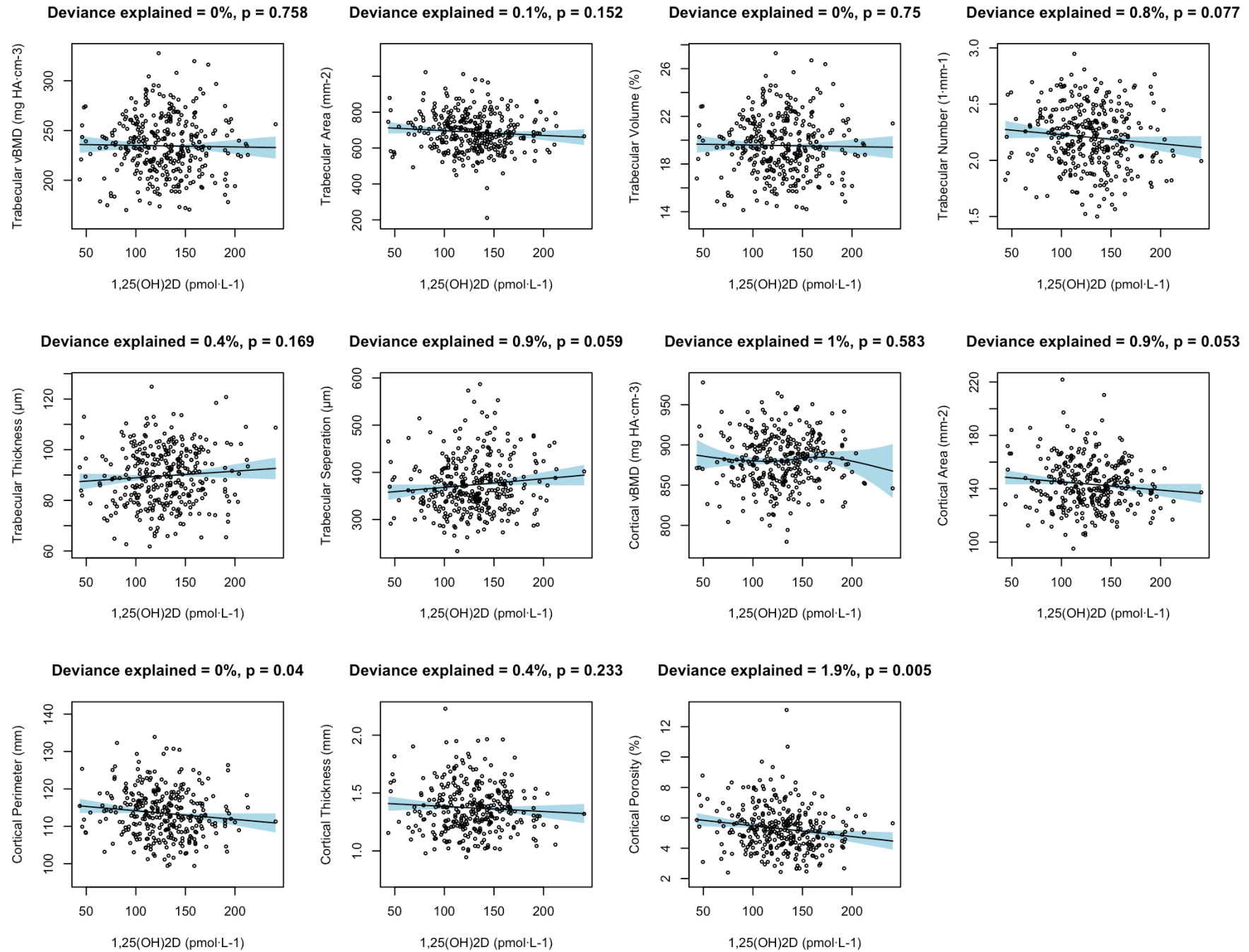
	Placebo (n = 33)				Vitamin D (n = 40)			
	Week 1	Week 12	Mean Difference [95% CI]	Hedges' g	Week 1	Week 12	Mean Difference [95% CI]	Hedges' g
Total vBMD (mgHA·cm ⁻³)	347 $\pm$ 45	347 $\pm$ 42	0 [-6, 5]	-0.02	359 $\pm$ 47	359 $\pm$ 46	0 [-3, 3]	0.00
Trabecular vBMD (mgHA·cm ⁻³)	229 $\pm$ 29	232 $\pm$ 45	3 [-9, 15]	0.08	235 $\pm$ 25	233 $\pm$ 32	-2 [-10, 6]	-0.08
Trabecular Area (mm ²)	689 $\pm$ 130	687 $\pm$ 129 ^a	-1 [-2, 0]	-0.47	672 $\pm$ 139	672 $\pm$ 139 ^a	0 [-1, 1]	-0.11
Trabecular Volume (%)	19.0 $\pm$ 2.3	18.9 $\pm$ 2.2	-0.1 [-0.3, 0.1]	-0.20	19.7 $\pm$ 2.1	19.7 $\pm$ 2.2	0.0 [-0.2, 0.2]	0.01
Trabecular Number (1·mm)	2.21 $\pm$ 0.28	2.23 $\pm$ 0.26	0.01 [-0.07, 0.10]	0.06	2.27 $\pm$ 0.29	2.35 $\pm$ 0.28	0.08 [0.00, 0.15]	0.33
Trabecular Thickness (μm)	87 $\pm$ 13	85 $\pm$ 11 ^a	-1 [-4, 2]	-0.13	87 $\pm$ 12	84 $\pm$ 12 ^a	-3 [-6, 0]	-0.35
Trabecular Separation (μm)	372 $\pm$ 52	360 $\pm$ 76 ^a	-12 [-36, 11]	-0.19	354 $\pm$ 63	330 $\pm$ 69 ^a	-24 [-44, -4]	-0.37
Cortical vBMD (mgHA·cm ⁻³)	895 $\pm$ 33	892 $\pm$ 26	0 [-6, 6]	0.00	899 $\pm$ 43	901 $\pm$ 41	-2 [-7, 3]	-0.14
Cortical Area (mm ²)	147 $\pm$ 24	149 $\pm$ 24 ^a	2 [0, 3]	0.46	146 $\pm$ 25	146 $\pm$ 25 ^a	1 [-1, 2]	0.16
Cortical Thickness (mm) ¹ ^a	1.30 $\pm$ 0.22	1.31 $\pm$ 0.21 ^a	0.02 [0.00, 0.03]	0.44	1.31 $\pm$ 0.27	1.31 $\pm$ 0.26 ^a	0.00 [-0.01, 0.01]	0.03
Cortical Porosity (%)	4.69 $\pm$ 1.24	4.49 $\pm$ 1.39	-0.15 [-0.53, 0.23]	-0.15	4.57 $\pm$ 1.46	4.70 $\pm$ 1.46	0.11 [-0.09, 0.30]	0.20
Stiffness (kN·mm ⁻¹)	274 $\pm$ 42	274 $\pm$ 39	1 [-5, 7]	0.07	277 $\pm$ 38	273 $\pm$ 40	-3 [-8, 2]	-0.21
Failure Load (kN)	13.76 $\pm$ 2.01	13.74 $\pm$ 1.93	0.06 [-0.17, 0.28]	0.10	13.89 $\pm$ 1.86	13.74 $\pm$ 1.89	-0.11 [-0.30, 0.07]	-0.20

^ap < 0.05 vs week 1 (main effects of time, both groups pooled).

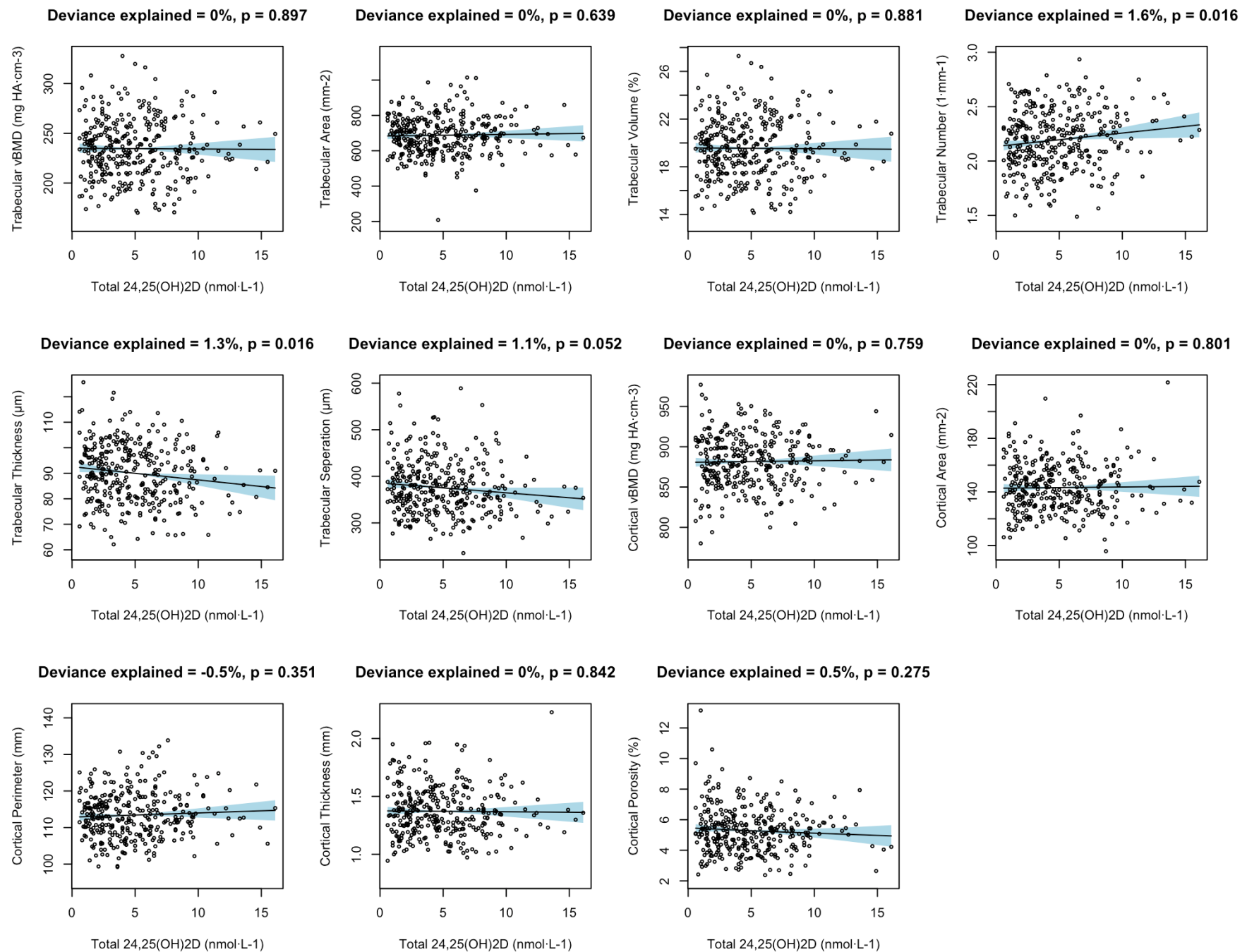
vBMD, volumetric bone mineral density.



736 **Figure 1.** Associations between serum total 25-hydroxyvitamin D (25(OH)D) and tibial trabecular and cortical volumetric bone mineral density, geometry, and
737 microarchitecture generated by generalized additive models controlling for age, height, lean mass, fat mass, exercise volume, and smoking. Blue shaded areas
738 represent 95% CIs.



740 **Figure 2.** Associations between serum 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) and tibial trabecular and cortical volumetric bone mineral density, geometry, and
741 microarchitecture generated by generalized additive models controlling for age, height, lean mass, fat mass, exercise volume, and smoking. Blue shaded lines
742 represent 95% CIs.



744 **Figure 3.** Associations between serum total 24,25-dihydroxyvitamin D (24,25(OH)₂D) and tibial trabecular and cortical volumetric bone mineral density,
745 geometry, and microarchitecture generated by generalized additive models controlling for age, height, lean mass, fat mass, exercise volume, and smoking. Blue
746 shaded lines represent 95% CIs.

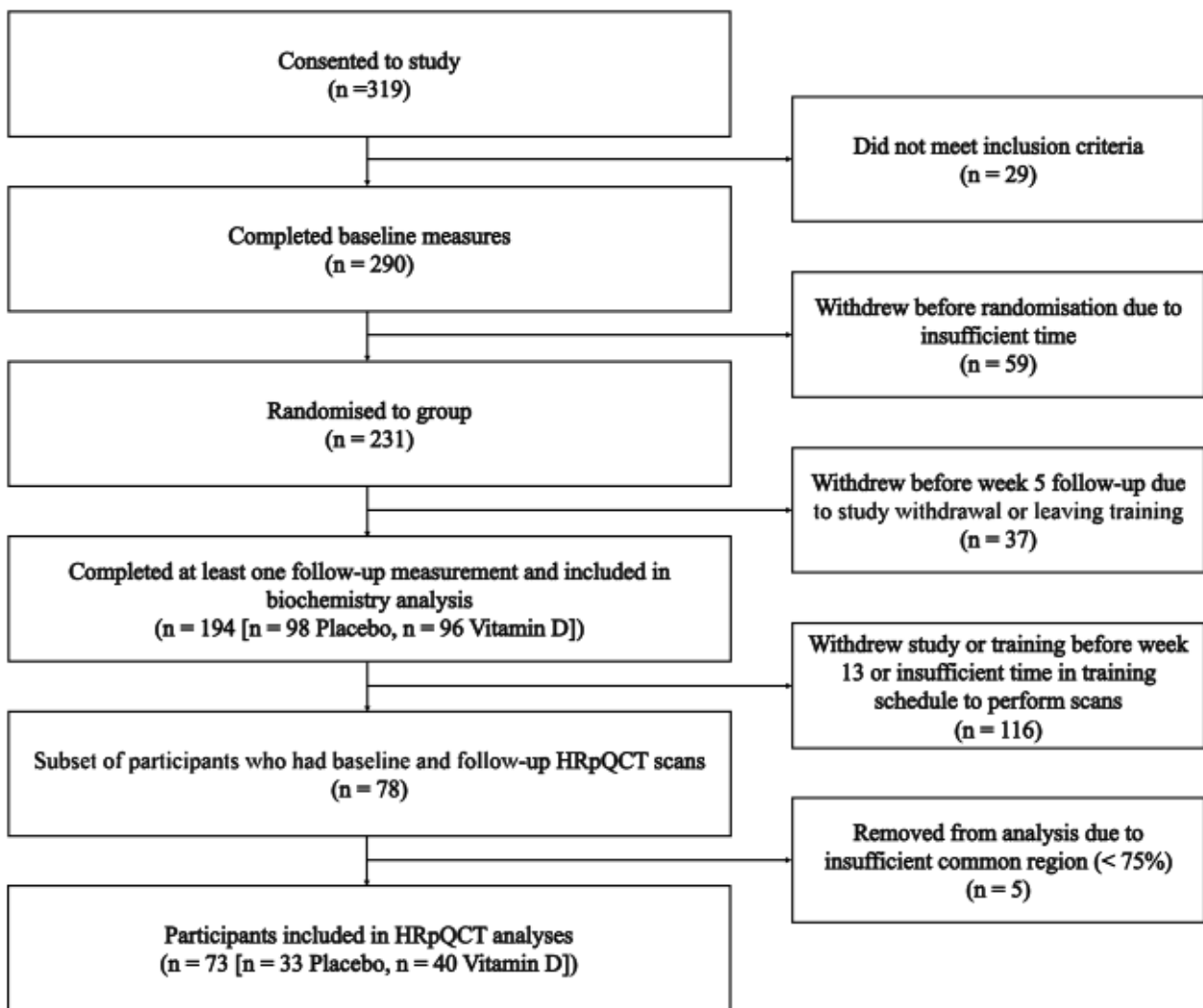


Figure 4. Participant flow through Study 2.

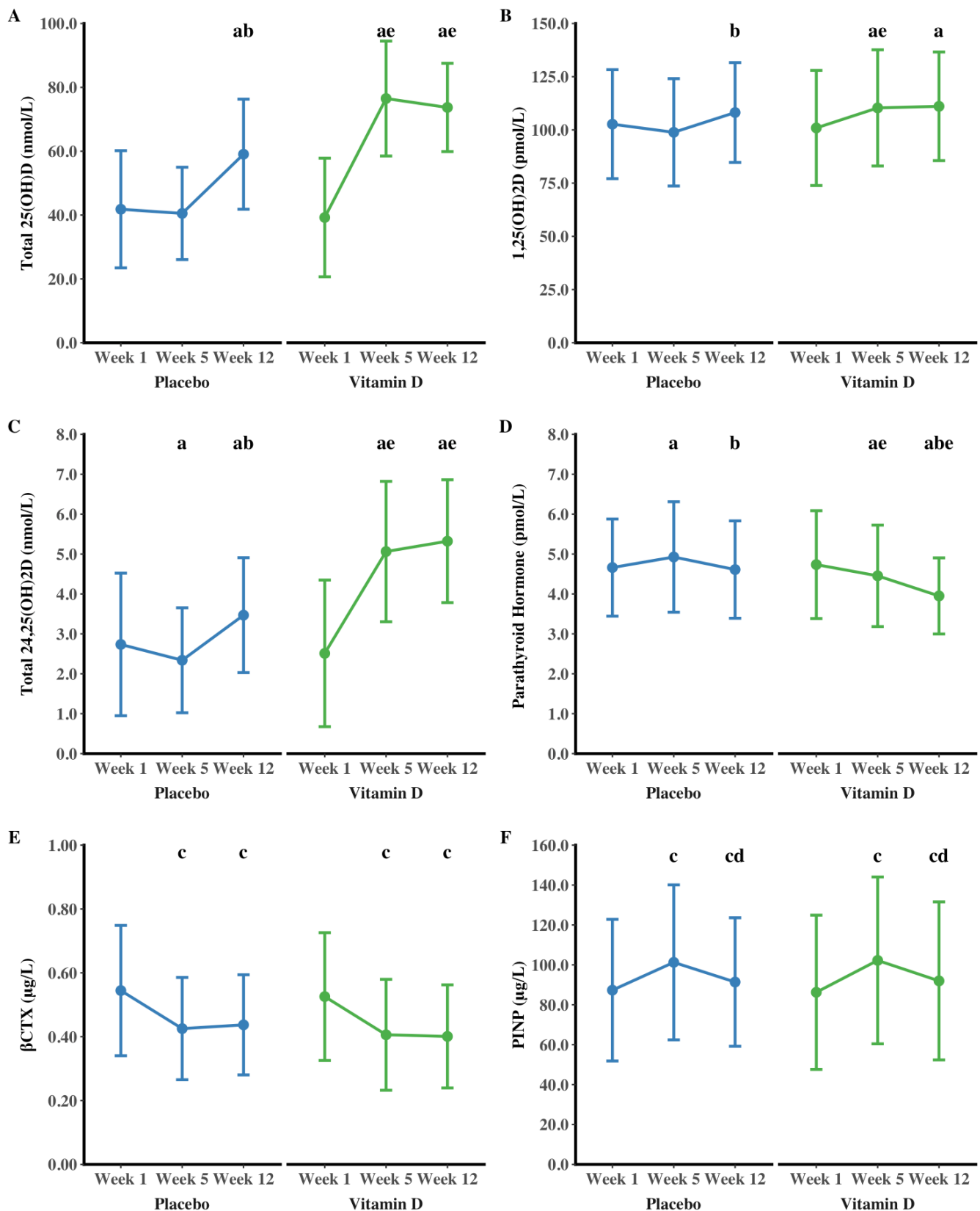
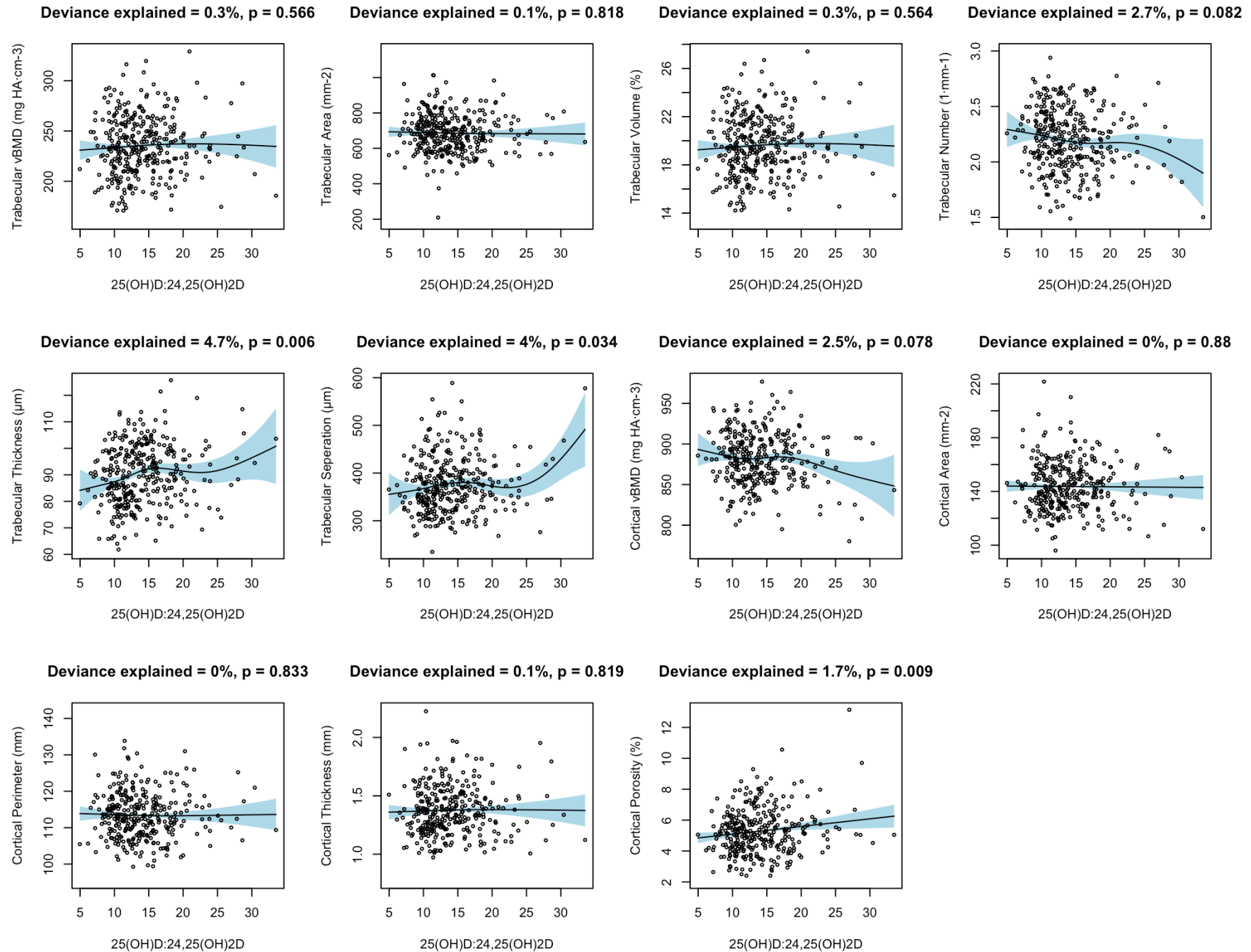
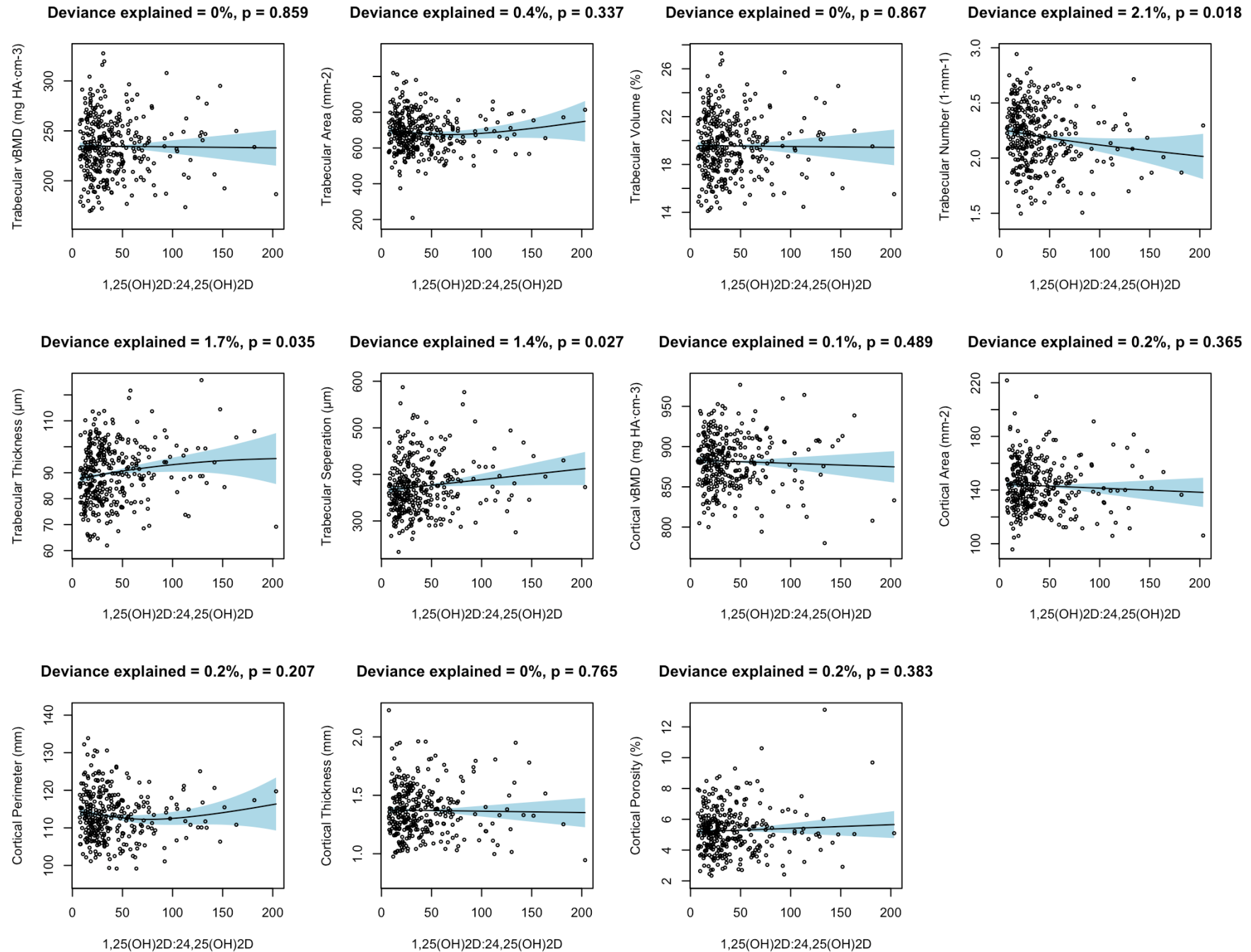


Figure 5. Circulating vitamin D metabolites and markers of bone metabolism in response to 12 weeks of military training in men supplemented with placebo or vitamin D. Data are mean $\pm$ SD.

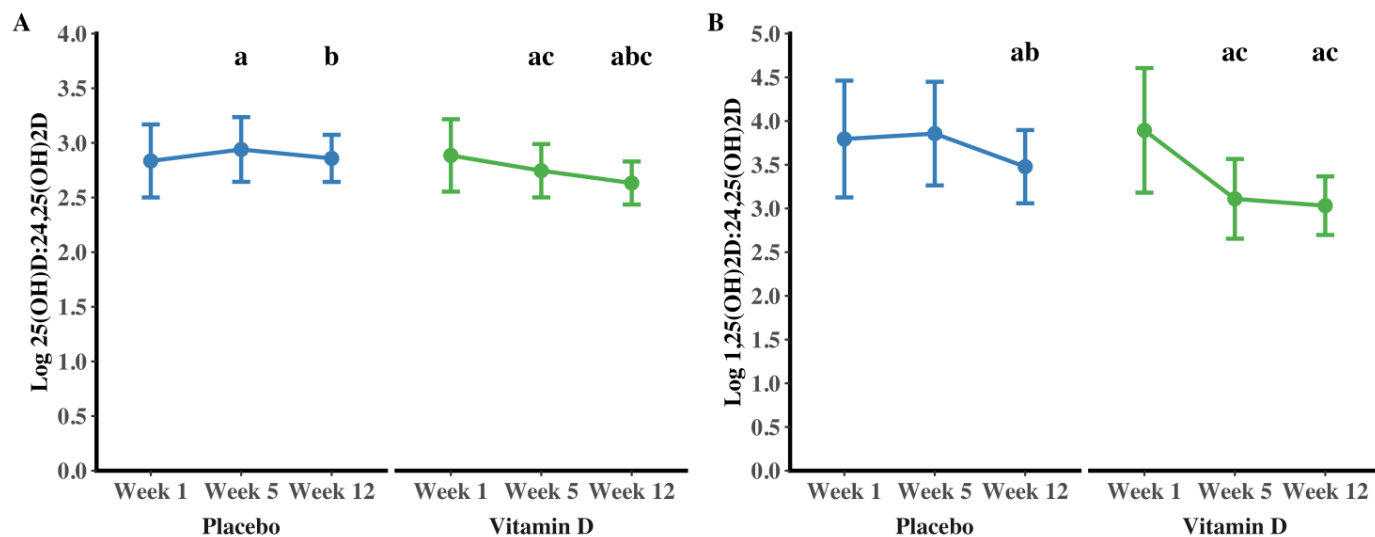
^ap < 0.05 vs week 1 (post-hoc within group); ^bp < 0.05 vs week 5 (post-hoc within group); ^cp < 0.05 vs week 1 (main effects of time, both groups pooled); ^dp < 0.05 vs week 5 (main effects of time, both groups pooled); ^ep < 0.05 vs placebo at the same time-point.



755 **Supplementary Figure 1.** Associations between VMR 1 (25(OH)D:24,25(OH)₂D) and tibial trabecular and cortical volumetric bone mineral density, geometry,
756 and microarchitecture generated by generalized additive models controlling for age, height, lean mass, fat mass, exercise volume, and smoking. Blue shaded lines
757 represent 95% CIs.

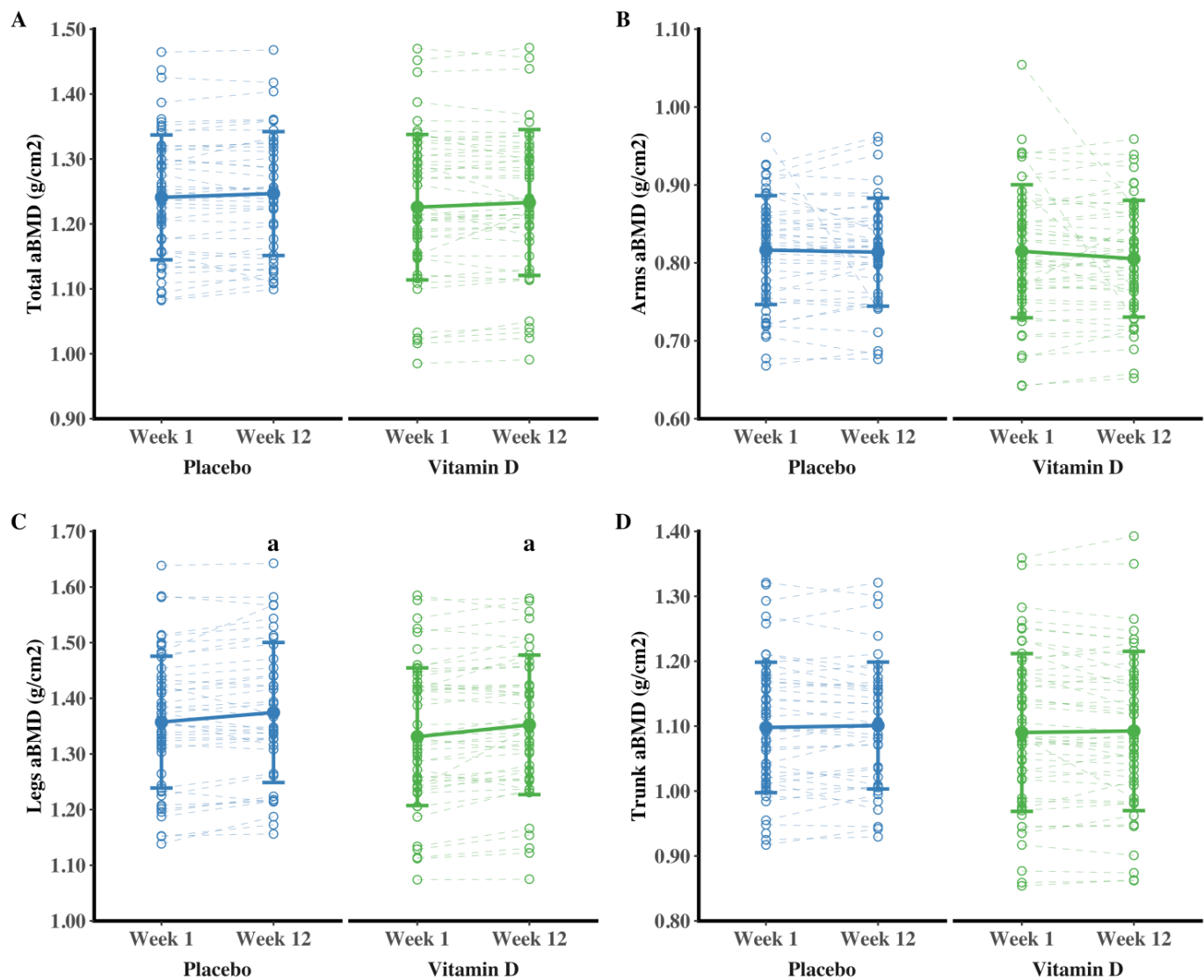


759 **Supplementary Figure 2.** Associations between VMR 2 (1,25(OH)₂D:24,25(OH)₂D) and tibial trabecular and cortical volumetric bone mineral density,
760 geometry, and microarchitecture generated by generalized additive models controlling for age, height, lean mass, fat mass, exercise volume, and smoking. Blue
761 shaded lines represent 95% CIs.



Supplementary Figure 3. Circulating vitamin D metabolite ratios in response to 12 weeks military training in men supplemented with placebo or vitamin D. Data are mean $\pm$ SD.

^ap < 0.05 vs week 1 (post-hoc within group); ^bp < 0.05 vs week 5 (post-hoc within group); ^cp < 0.05 vs placebo at the same time-point.



Supplementary Figure 4. Whole-body and regional areal bone mineral density in response to 12 weeks military training in men supplemented with placebo or vitamin D.

^ap < 0.05 vs week 1 (main effects of time, both groups pooled).