Iron status is associated with tibial structure and vitamin D metabolites in healthy young men

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Competing Interests

The authors have no competing interests to declare.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract

The influence of iron on collagen synthesis and vitamin D metabolism has implications for bone health. This cross-sectional observational study investigated associations between markers of iron status and tibial structure, vitamin D metabolites, and circulating biochemical markers of bone metabolism in young healthy men. A total of 343 male British Army recruits participated (age 22 ± 3 y, height 1.77 ± 0.06 m, body mass 75.5 ± 10.1 kg). Circulating biochemical markers of iron status, vitamin D metabolites, and bone metabolism, and tibial structure and density by high-resolution peripheral quantitative computed tomography scans (HRpQCT) were measured in participants during week 1 of basic military training. Associations between markers of iron status and HRpQCT outcomes, bone metabolism, and vitamin D metabolites were tested, controlling for age, height, lean body mass, and childhood exercise volume. Higher ferritin was associated with higher total, trabecular, and cortical volumetric bone mineral density, trabecular volume, cortical area and thickness, stiffness, and failure load (all $p \leq 0.037$). Higher soluble transferrin receptor (sTfR) was associated with lower trabecular number, and higher trabecular thickness and separation, cortical thickness, and cortical pore diameter (all $p \leq 0.033$). Higher haemoglobin was associated with higher cortical thickness ($p = 0.043$). Higher ferritin was associated with lower βCTX, PINP, total 25(OH)D, and total $24,25(OH)_2D$, and higher $1,25(OH)_2D$:$24,25(OH)_2D$ ratio (all $p \leq 0.029$). Higher sTfR was associated with higher PINP, total 25(OH)D, and total $24,25(OH)_2D$ (all $p \leq 0.025$). The greater density, size, and strength of the tibia, and lower circulating concentrations of markers of bone resorption and formation with better iron stores (higher ferritin) are likely as a result of the direct role of iron in collagen synthesis.

Key Words: Bone; Military; Musculoskeletal Injury; Nutrition; Stress Fracture.

1. Introduction

Iron is a trace element that contributes to physiological function by incorporation into enzymes and proteins involved in energetic pathways and oxygen storage and transport—including myoglobin and haemoglobin (>75% total body iron) [1]. Accordingly, iron status is important for physical and cognitive performance [2]. Beyond these classical roles of iron in energy metabolism and oxygen storage and transport, iron plays an important role in bone health. Iron is an essential cofactor for the prolyl- and lysyl-hydroxylase enzymes, key enzymes in collagen synthesis [3–7]. Iron is also essential for vitamin D metabolism. Vitamin D is hydroxylated to 25-hydroxyvitamin D (25(OH)D) in the liver, then to the most biologically active form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) in the kidney, before hydroxylation to 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) [8]. Vitamin D hydroxylation reactions are catalysed by cytochrome P450 enzymes of which iron is an essential cofactor [8]. Iron deficiency causes hypoxia, which when combined with reduced cellular iron, reduces prolyl hydroxylase activity, increases hypoxia-inducible transcription factor activity, resulting in suppressed osteoclastogenesis and enhanced osteoclast activity [3,4,7].

Iron status is determined by circulating levels of ferritin, transferrin saturation, soluble transferrin receptor (sTfR), erythrocyte distribution width (RDW), mean corpuscular volume (MCV), and haemoglobin [2,9–11]. Iron deficiency exists on a continuum but is defined as low iron stores (low ferritin) before haemoglobin levels are affected, and iron deficiency anaemia is defined as low iron stores and low haemoglobin [1,2]. There are several criteria for defining iron deficiency, which complicates the interpretation of iron status. The World Health Organization defines iron deficiency as ferritin < 15 $\mu g \cdot dL^{-1}$ for men and women and anaemia as haemoglobin < 12 $g \cdot dL^{-1}$ for women and < 13 $g \cdot dL^{-1}$ for men [11]. Iron status has been associated with numerous bone outcomes. Higher dietary iron intake was positively associated

with whole-body, lumbar spine, and femur areal bone mineral density (aBMD) in postmenopausal women [12]. Iron deficiency and / or lower ferritin has been associated with increased osteoporosis risk in older adults [13,14] and lower lumbar spine and femur aBMD in older men [15]. Lower ferritin was associated with higher concentrations of circulating concentrations of bone resorption markers [16] and treatment of iron deficiency with iron supplements decreased circulating concentrations of markers of bone resorption and formation [17] in premenopausal women. Animal studies show that restricting iron intake decreases femur and tibial volumetric bone mineral density (vBMD), cortical thickness and area, and strength [18,19], lumbar spine trabecular volume and number, and increases trabecular separation [19]. The association between iron status and trabecular and cortical bone in young adults has yet to be explored but these data would provide important evidence for the role of iron status in human bone structure.

Military recruits are at a high risk of tibial bone stress injury, particularly in those undergoing the most arduous infantry training [20,21]. Basic military training diminishes iron status in women [10,22–25] and men [25–28]. Better understanding of the iron status and tibial bone structure of young military recruits may provide important insight into reducing bone stress injury risk. We have previously reported an association between higher ferritin and lower circulating concentrations of markers of bone resorption and formation, and lower whole-body aBMD [29]. The primary aim of this study was to examine the association between iron status and tibial macro- and micro-structure measured by high-resolution peripheral quantitative computed tomography (HRpQCT) in male British Army infantry recruits, whose military training diminishes iron status [29] and results in a high risk of tibial bone stress injuries [20]. Secondary aims were to examine the associations between iron status and biochemical markers of bone and vitamin D metabolism. We hypothesised that better iron status would be associated

with higher tibial vBMD and size, lower circulating concentrations of bone resorption and formation, and higher concentrations of total 25(OH)D.

2. Materials and Methods

2.1 Participants

The study was advertised to new male British Army infantry trainees from January 2014 to July 2017 during week one of their basic training course at the Infantry Training Centre, Catterick. Women were excluded from infantry roles at the time of data collection and so only men were included. Participants had passed their military medical assessment and were declared free of any injury or health condition precluding military training. All procedures were approved by the Ministry of Defence Research Ethics Committee (ref: 165/Gen/10). Each participant had the study procedures and risks fully explained verbally and in writing. Written informed consent was obtained from all participants.

2.2 Study Design

This study was an observational cross-sectional study. These data present the bone outcomes from a larger study exploring micronutrient deficiencies and health and performance outcomes in military recruits [29–32]. All data were collected at the start (week 1) of basic military training before military training commenced. Participants were completing the 26-week British Army infantry basic training course or the 28-week British Army parachute regiment course. Tibial macro- and microstructure was measured by HRpQCT. Venous blood samples were drawn for the analysis of biochemical markers of iron status, biochemical markers of bone metabolism, and vitamin D metabolites. Body mass, height, and body composition by dual-energy X-ray absorptiometry (DXA) were measured. Participants self-reported their habitual exercise levels during the ages of 12 to 16 years using questionnaires.

2.3 Tibial Volumetric Bone Mineral Density, Geometry, and Microarchitecture

First generation high-resolution peripheral quantitative computed tomography (XtremeCT, Scanco Medical AG, Switzerland) was used to assess vBMD, geometry and microarchitecture of the ultra-distal tibia in the non-dominant leg. Leg dominance was self-determined and described to participants as the leg most likely used to kick a ball. A three-dimensional representation of 9.02 mm of the tibia in the axial direction was obtained from 110 CT slices with an isotropic voxel size of 82 μm. The leg of each participant was fitted into a carbon fibre shell and immobilised within the gantry of the scanner for the duration of the scan (2.8 min). A reference line was positioned at the tibial endplate with the first CT slice taken from 22.5 mm proximal to the reference line. Daily quality control scans were performed using the manufacturer issued phantom that contained rods of hydroxyapatite (HA). The quality of each HRpQCT scan was reviewed by a single operator according to manufacturer visual grading instructions and any scans judged to be poor quality were repeated. The methods used to process the data have been previously described [33–35]. The standard evaluation procedure provided by the manufacturer was used to derive the following outcome variables: total vBMD (mg HA·cm^{-3}), trabecular vBMD (mg HA·cm^{-3}), cortical vBMD (mg HA·cm^{-3}), trabecular area (mm^{-2}), trabecular bone volume fraction (%), number of trabeculae (1·mm^{-1}), trabecular thickness (mm^{-1}), trabecular separation (mm^{-1}), cortical area (mm^{-2}), cortical thickness (mm^{-1}), and cortical perimeter (mm^{-1}). Detailed analysis of cortical bone was performed using a semi-automated segmentation technique to determine cortical porosity (%) and average cortical pore diameter (mm^{-1}) [33,34]. Micro-finite element analysis was performed as described previously [36], to estimate the biomechanical properties under uniaxial compression, specifically stiffness (kN·mm^{-1}) and failure load (kN). All scans and evaluations were performed by a single investigator to ensure consistency. The coefficient of variation (CV) is ≤ 1.5% for vBMD, ≤

4.4% for trabecular microarchitecture, ≤ 1.5% for cortical thickness, ≤ 1.5% for cortical and trabecular area, and ≤ 6.2% for cortical porosity [33,35].

2.4 Blood Collection and Handling

A venous blood sample was collected either in the morning (~0900 to 1100 h) or early afternoon (~1300 to 1500 h) after participants had eaten breakfast (0600 to 0700 h) or lunch (1200 to 1300 h). Venous blood was drawn from a vein in the antecubital fossa and collected in serum and EDTA BD Vacutainer® 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 into universal tubes and stored at −80°C until analysis.

2.5 Biochemical Analyses

Haemoglobin, RDW, and MCV were measured in EDTA whole blood within 30 min of collection using the COULTER A^{C}·T diff 2 Analyzer (Beckman Coulter, California, USA). Plasma procollagen type 1 N-terminal propeptide (PINP), c-telopeptide cross-links of type 1 collagen (βCTX), parathyroid hormone (PTH), and serum ferritin were analysed by electro-chemiluminescence immunoassays (ECLIA) on the COBAS c601 platform (Roche Diagnostics, Mannheim, Germany). PINP inter-assay CV was < 3% between 20.0 and 600.0 $\mu g \cdot L^{-1}$ with a sensitivity of 8.0 $\mu g \cdot L^{-1}$. βCTX inter-assay CV was < 3% between 0.20 and 1.50 $\mu g \cdot L^{-1}$ with a sensitivity of 0.01 $\mu g \cdot L^{-1}$. PTH inter-assay CV was < 3.8% between 0.1 and 530.0 $pmol \cdot L^{-1}$. Ferritin inter-assay CV was < 4.2% between 0.5 and 2000.0 $\mu g \cdot L^{-1}$. Serum sTfR was measured by immunoturbidimetric assays performed on the COBAS c501 analyser (Roche Diagnostics, Mannheim, Germany). sTfR inter-assay CV was < 6.0% between 5.9 and 472.0 $nmol \cdot L^{-1}$. Serum samples were analysed for total 25(OH)D (sum of $25(OH)D_2$ and $25(OH)D_3$)

and total 24,25$(OH)_2D$ (sum of 24,25$(OH)_2D_2$ and 24,25$(OH)_2D_3$) by high-performance liquid chromatography tandem mass spectrometry using a Micromass Quattro Ultima Pt electrospray ionisation mass spectrometer [37]. The 25$(OH)D_3$ and 25$(OH)D_2$ 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 measurement ranges of the assays were 0.1 to 200.0 nmol·L^{-1} for 25$(OH)D_2$ and 25$(OH)D_3$, 0.8 to 25.0 nmol·L^{-1} for 24,25$(OH)_2D_2$, 0.1 to 25.0 nmol·L^{-1} for 24,25$(OH)_2D_3$, and 12 to 480 pmol·L^{-1} for 1,25$(OH)_2D$. The mean CV for intra-assay imprecision across the measuring range of the assays was 4.9% for 25$(OH)D_2$, 8.3% for 25$(OH)D_3$, 7.7% for 24,25$(OH)_2D_2$, 9.0% for 24,25$(OH)_2D_3$, and 7.4% for 1,25$(OH)_2D$. The cumulative inter-assay CVs were ≤ 7.4% for 25$(OH)D_2$, ≤ 9.6% for 25$(OH)D_3$, ≤ 10.6% for 24,25$(OH)_2D_2$, ≤ 8.9% for 24,25$(OH)_2D_3$, and ≤ 9.3% for 1,25$(OH)_2D$. The vitamin D metabolite ratios 25(OH)D:24,25$(OH)_2D$ and 1,25$(OH)_2D$:24,25$(OH)_2D$ were calculated as described previously [30,38,39]. All biochemical analyses (excluding haemoglobin, RDW, and MCV analyses) were undertaken by the Good Clinical Laboratory Practice and Vitamin D External Quality Assessment Scheme (DEQAS) certified Bioanalytical Facility at the University of East Anglia, Norwich, UK. Our 25(OH)D and 24,25$(OH)_2D$ assays showed < 6% accuracy bias against Centers for Disease Control and Prevention's reference method on the DEQAS, and < 9% bias against the method-specific mean for 1,25$(OH)_2D$. We met the certification performance standards set by DEQAS when the analyses were performed.

2.6 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%.

2.7 Statistical Analyses

These data were secondary analyses [30–32] and so no *a priori* sample size was calculated. All data were analysed using the R programming language (*v*.4.2.2). Multiple linear regression was used to test the association between each marker of iron status with tibial structure (HRpQCT outcomes), markers of bone metabolism (βCTX, P1NP, and PTH), and markers of vitamin D metabolism (total 25(OH)D, $1,25(OH)_2D$, total $24,25(OH)_2D$, 25(OH)D:$24,25(OH)_2D$, and $1,25(OH)_2D$:$24,25(OH)_2D$), controlling for age, height, lean body mass, and childhood exercise volume. Each marker of iron status was entered separately into each multiple linear regression. Variance and normality of the residuals were checked visually by plotting the residuals against the fitted values and from Q-Q plots. Data are presented as unstandardised coefficients. Significance was accepted as $p \leq 0.05$.

3. Results

3.1 Participants

A total of 1332 male infantry recruits volunteered to participate in this study. A randomly selected convenience sample of 343 participants were selected from this study pool for HRpQCT measurements (Figure 1, Table 1). A post-hoc power calculation revealed that 343 participants in a model with five coefficients (minus the intercept) were sufficient to detect an effect size of $f^2 = 0.04$ (small effect) with an $\alpha = 0.05$ and a $1 - \beta = 0.80$ (*pwr* package *v*1.3-0). The demographics of the total sample has been published previously [29] and the demographics of those selected for HRpQCT measurements in this study were very similar. Participants were recruited throughout the year (spring n = 59, summer n = 104, autumn n = 123, winter n = 57). Based on the World Health Organization definition [11] for iron deficiency, one participant

(0.3%) met the criteria for iron deficiency (ferritin < 15 μg·dL^{-1} and haemoglobin ≥ 13 g·dL^{-1}) and four participants (1.4%) met the criteria for anaemia (haemoglobin < 13 g·dL^{-1}).

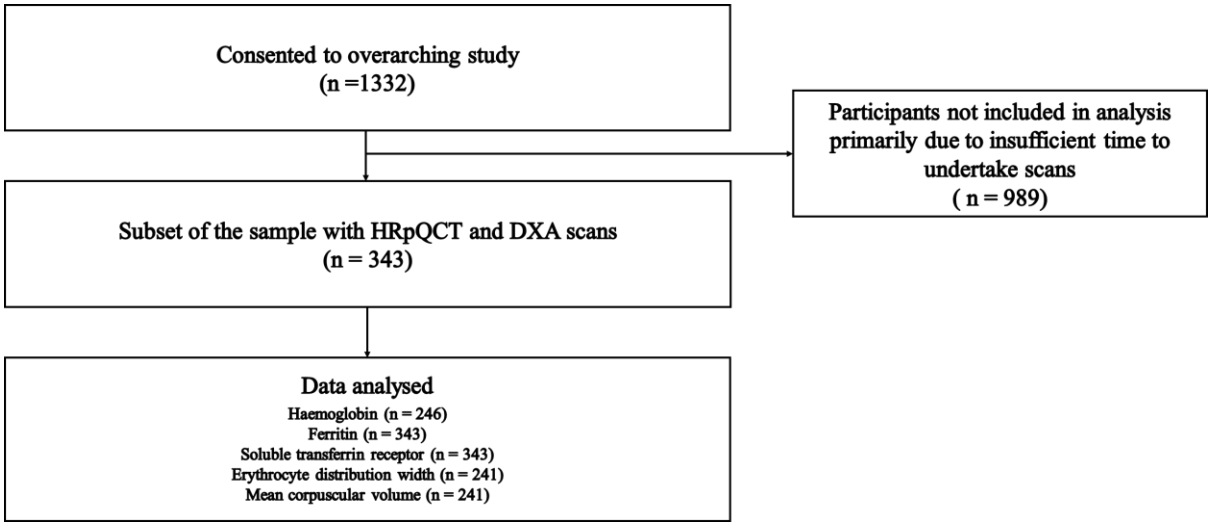

Figure 1. Participant flow through the study.

231 **Table 1.** Participant demographics.

	Mean ± SD or median [interquartile range]
Demographics	
Age (years)	22 ± 3
Body Mass (kg)	75.5 ± 10.1
Height (m)	1.77 ± 0.06
Body Mass Index ($kg \cdot m^{-2}$)	24.0 ± 2.7
Habitual Exercise Volume ($mins \cdot week^{-1}$)	360 [208, 360]
Body Composition	
Fat Mass (kg)	14.3 ± 5.6
Body Fat (%)	18.8 ± 5.5
Lean Body Mass (kg)	57.3 ± 6.4
Whole-body aBMD ($g \cdot cm^{-2}$)	1.23 ± 0.11
Vitamin D Metabolites	
Total 25(OH)D ($nmol \cdot L^{-1}$)	60.6 ± 29.2
$1,25(OH)_2D$ ($pmol \cdot L^{-1}$)	129 ± 34
Total $24,25(OH)_2D$ ($nmol \cdot L^{-1}$)	4.9 ± 3.1
$25(OH)D:24,25(OH)_2D$	14.0 ± 5.0
$1,25(OH)_2D:24,25(OH)_2D$	39.6 ± 31.0
Markers of Bone Metabolism	
βCTX ($\mu g \cdot L^{-1}$)	0.49 ± 0.20
PINP ($\mu g \cdot L^{-1}$)	98.7 ± 44.1
PTH ($pmol \cdot L^{-1}$)	3.7 ± 1.2
Markers of Iron Status	
Ferritin ($\mu g \cdot L^{-1}$)	97 ± 59
sTfR ($nmol \cdot L^{-1}$)	26.7 ± 8.2
Haemoglobin ($g \cdot dL^{-1}$)	15.1 ± 0.9
MCV (fL)	89.6 ± 3.4
RDW (%)	13.0 ± 0.7
Tibial Structure	
Total Area (mm^{-2})	846 ± 142
Total vBMD (mg $HA \cdot cm^{-3}$)	347 ± 48

	Mean ± SD or median [interquartile range]
Trabecular Area (mm^{-2})	699 ± 145
Trabecular vBMD (mg HA·cm^{-3})	229 ± 31
Trabecular Volume (%)	19.0 ± 2.6
Trabecular Number ($1 \cdot mm^{-1}$)	2.19 ± 0.30
Trabecular Thickness (μm^{-1})	88 ± 12
Trabecular Separation (μm^{-1})	378 ± 63
Cortical Area (mm^{-2})	139 ± 20
Cortical vBMD (mg HA·cm^{-3})	887 ± 38
Cortical Perimeter (mm^{-1})	114 ± 9
Cortical Porosity (%)	4.80 ± 1.61
Cortical Thickness (mm^{-1})	1.32 ± 0.24
Cortical Pore Diameter (mm^{-1})	0.165 ± 0.016
Stiffness ($kN \cdot mm^{-1}$)	281 ± 42
Failure Load (kN)	14.08 ± 2.03

$1,25(OH)_2D$, 1,25-dihydroxyvitamin D; aBMD, areal bone mineral density; βCTX, c-telopeptide cross-links of type 1 collagen; MCV, mean corpuscular volume; PINP, procollagen type 1 N-terminal propeptide; PTH, parathyroid hormone; RDW, erythrocyte distribution width; sTfR, soluble transferrin receptor; Total 25(OH)D, total 25-hydroxyvitamin D; Total 24,25(OH)2D, total 24,25-dihydroxyvitamin D; vBMD, volumetric bone mineral density.

Missing data: βCTX = 1, PINP = 1, PTH = 1, Haemoglobin = 97, MCV = 102, RDW = 102, 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, Cortical Pore Diameter = 16, Stiffness = 12, Failure Load = 12

3.2 Associations Between Iron Status and Bone Structure

Associations between markers of iron status and total and trabecular bone structure are shown in Table 2. Higher ferritin was associated with higher total vBMD, trabecular vBMD, and higher trabecular volume. Higher sTfR was associated with lower trabecular number, higher trabecular thickness, and higher trabecular separation. Higher RDW was associated with higher trabecular thickness. Haemoglobin and MCV were not associated with total or trabecular bone structure. Associations between markers of iron status and cortical bone structure and estimated

mechanical strength are shown in Table 3. Higher ferritin was associated with higher cortical area, cortical vBMD, cortical thickness, stiffness, and failure load. Higher sTfR was associated with higher cortical thickness and cortical pore diameter. Higher haemoglobin was associated with higher cortical thickness. Higher RDW was associated with higher cortical pore diameter. MCV was not associated with cortical bone structure or estimated mechanical strength.

Table 2. Associations between iron status and total and trabecular bone structure.

Marker*	**Tt.Area (mm^{-2})** Coefficient (95% CI)	p	**Tt.vBMD (mg HA·cm^{-3})** Coefficient (95% CI)	p	**Tb.Area (mm^{-2})** Coefficient (95% CI)	p	**Tb.vBMD (mg HA·cm^{-3})** Coefficient (95% CI)	p	**Tb.BV/TV (%)** Coefficient (95% CI)	p	**Tb.N ($1 \cdot mm^{-1}$)** Coefficient (95% CI)	p	**Tb.Th (μm^{-1})** Coefficient (95% CI)	p	**Tb.Sp (μm^{-1})** Coefficient (95% CI)	p
Ferritin ($\mu g \cdot L^{-1}$)	−0.049 (−0.240, 0.143)	0.616	0.108 (0.025, 0.191)	**0.011**	−0.101 (−0.306, 0.104)	0.332	0.058 (0.003, 0.113)	**0.037**	0.005 (0.000, 0.010)	**0.034**	0.000 (0.000, 0.001)	0.230	0.008 (−0.014, 0.029)	0.474	−0.091 (−0.199, 0.017)	0.100
sTfR ($nmol \cdot L^{-1}$)	−0.867 (−2.232, 0.497)	0.212	0.467 (−0.130, 1.063)	0.125	−1.202 (−2.660, 0.256)	0.106	0.098 (−0.296, 0.493)	0.624	0.008 (−0.025, 0.041)	0.635	−0.005 (−0.008, −0.001)	**0.013**	0.231 (0.080, 0.381)	**0.003**	0.839 (0.067, 1.610)	**0.033**
Haemoglobin ($g \cdot dL^{-1}$)	−9.865 (−25.428, 5.698)	0.213	2.758 (−4.065, 9.581)	0.427	−12.510 (−29.232, 4.211)	0.142	−1.024 (−5.327, 3.278)	0.640	−0.086 (−0.445, 0.273)	0.637	−0.028 (−0.068, 0.012)	0.171	0.809 (−0.880, 2.498)	0.346	6.003 (−2.235, 14.240)	0.152
MCV (fL)	0.127 (−3.955, 4.209)	0.951	−0.594 (−2.387, 1.199)	0.515	0.538 (−3.852, 4.928)	0.809	−0.795 (−1.925, 0.335)	0.167	−0.066 (−0.160, 0.028)	0.168	−0.002 (−0.013, 0.008)	0.685	−0.204 (-0.647, 0.239)	0.366	0.582 (−1.605, 2.769)	0.601
RDW (%)	−7.932 (−27.693, 11.830)	0.430	3.165 (−5.525, 11.854)	0.474	−7.790 (−29.052, 13.472)	0.471	2.530 (−2.960, 8.020)	0.365	0.208 (−0.250, 0.665)	0.373	−0.043 (−0.095, 0.008)	0.098	2.778 (0.657, 4.899)	**0.010**	7.364 (−3.203, 17.931)	0.171

*controlling for age, height, lean body mass, and habitual exercise volume.

MCV, mean corpuscular volume; RDW, erythrocyte distribution width; sTfR, soluble transferrin receptor; Tb.Area, trabecular area; Tb.BV/TV, trabecular bone volume; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; Tb.vBMD, trabecular volumetric bone mineral density; Tt.Area, total area; Tt.vBMD, total volumetric bone mineral density.

Table 3. Associations between iron markers and cortical bone structure and estimated mechanical strength.

	Ct.Area (mm^{-2})		**Ct.vBMD (mg HA·cm^{-3})**		**Ct.Pm (mm^{-1})**		**Ct.Po (%)**		**Ct.Th (mm^{-1})**		**Ct.Po.Dm (μm^{-1})**		**Stiffness ($kN \cdot mm^{-1}$)**		**Failure Load (kN)**	
Marker*	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p	Coefficient (95% CI)	p
Ferritin ($\mu g \cdot L^{-1}$)	0.038 (0.004, 0.072)	**0.031**	0.069 (0.007, 0.132)	**0.029**	−0.003 (−0.015, 0.009)	0.634	0.001 (−0.002, 0.004)	0.435	0.000[a] (0.000, 0.001)	**0.023**	0.025 (−0.005, 0.054)	0.099	0.084 (0.022, 0.147)	**0.009**	0.004 (0.001, 0.007)	**0.012**
sTfR ($nmol \cdot L^{-1}$)	0.230 (−0.013, 0.474)	0.064	0.056 (−0.388, 0.500)	0.803	−0.042 (−0.130, 0.045)	0.344	0.002 (−0.018, 0.022)	0.870	0.003 (0.001, 0.006)	**0.019**	0.242 (0.035, 0.449)	**0.022**	0.301 (−0.155, 0.757)	0.195	0.011 (−0.011, 0.032)	0.339
Haemoglobin ($g \cdot dL^{-1}$)	1.460 (−1.303, 4.222)	0.299	1.687 (−3.596, 6.969)	0.530	−0.849 (−1.838, 0.140)	0.092	0.045 (−0.179, 0.270)	0.692	0.032 (0.001, 0.063)	**0.043**	0.923 (−1.561, 3.406)	0.465	1.991 (−3.027, 7.008)	0.435	0.067 (−0.171, 0.306)	0.578
MCV (fL)	−0.472 (−1.207, 0.263)	0.207	−0.418 (−1.814, 0.978)	0.556	0.110 (−0.153, 0.372)	0.411	0.017 (−0.043, 0.076)	0.584	−0.004 (−0.012, 0.004)	0.338	0.391 (−0.254, 1.037)	0.234	−0.726 (−2.055, 0.604)	0.283	−0.030 (−0.093, 0.033)	0.353
RDW (%)	−3.156 (−7.012, 0.700)	0.108	0.844 (−6.501, 8.190)	0.821	−0.500 (−1.875, 0.875)	0.474	−0.085 (−0.399, 0.229)	0.594	−0.012 (−0.056, 0.032)	0.590	4.710 (1.363, 8.057)	**0.006**	0.976 (−5.590, 7.541)	0.770	0.011 (−0.301, 0.322)	0.945

*controlling for age, height, lean body mass, and habitual exercise volume.
a, coefficient = 0.0005

Ct.Area, cortical area; Ct.vBMD, cortical volumetric bone mineral density; Ct.Pm, cortical perimeter; Ct.Po, cortical porosity; Ct.Po.Dm, cortical pore diameter; Ct.Th, cortical thickness; MCV, mean corpuscular volume; RDW, erythrocyte distribution width; sTfR, soluble transferrin receptor.

256 *3.3 Associations Between Iron Status and Bone Metabolism*

257 Associations between markers of iron status and markers of bone and vitamin D metabolism
258 are shown in Table 4. Examination of the residuals revealed models with total $24,25(OH)_2D$
259 and $1,25(OH)_2D$:$24,25(OH)_2D$ as a response variable had a skewed distribution and so these
260 response variables were log transformed. Higher ferritin was associated with lower βCTX,
261 PINP, total 25(OH)D, and log total $24,25(OH)_2D$, and higher log $1,25(OH)_2D$:$24,25(OH)_2D$; a
262 10 $\mu g \cdot L^{-1}$ higher circulating concentration of ferritin was associated with a 0.04 $\mu g \cdot L^{-1}$, 1.9
263 $\mu g \cdot L^{-1}$, 0.8 $nmol \cdot L^{-1}$, and 0.02 $nmol \cdot L^{-1}$ lower circulating concentration of βCTX, PINP, total
264 25(OH)D, and log total $24,25(OH)_2D$, respectively. Higher sTfR was associated with higher
265 PINP, total 25(OH)D, and log total $24,25(OH)_2D$; a 1 $nmol \cdot L^{-1}$ higher circulating concentration
266 of sTfR was associated with a 0.6 $\mu g \cdot L^{-1}$, 0.5 $nmol \cdot L^{-1}$, and 0.01 $nmol \cdot L^{-1}$ higher circulating
267 concentration of PINP, total 25(OH)D, log total $24,25(OH)_2D$, respectively. Higher
268 haemoglobin was associated with lower $1,25(OH)_2D$; a 1 $g \cdot dL^{-1}$ higher circulating
269 concentration of haemoglobin was associated with a 6 $pmol \cdot L^{-1}$ lower circulating concentration
270 of $1,25(OH)_2D$. Higher MCV was associated with lower βCTX; a 10 fL higher MCV was
271 associated with a 0.07 $\mu g \cdot L^{-1}$ lower circulating concentration of βCTX. Higher RDW was
272 associated with higher $1,25(OH)_2D$; a 1% higher RDW was associated with a 10 $pmol \cdot L^{-1}$
273 higher circulating concentration of $1,25(OH)_2D$.

Table 4. Associations between iron status and biochemical markers of bone metabolism and vitamin D metabolites.

Marker*	**βCTX (μg·L^{-1})** Coefficient (95% CI)	p	**PINP (μg·L^{-1})** Coefficient (95% CI)	p	**PTH (pmol·L^{-1})** Coefficient (95% CI)	p	**Total 25(OH)D (nmol·L^{-1})** Coefficient (95% CI)	p	**1,25(OH)$_2$D (pmol·L^{-1})** Coefficient (95% CI)	p	**Log 24,25(OH)$_2$D (nmol·L^{-1})** Coefficient (95% CI)	p	**25(OH)D: 24,25(OH)$_2$D** Coefficient (95% CI)	p	**Log 1,25(OH)$_2$D: 24,25(OH)$_2$D** Coefficient (95% CI)	p
Ferritin (μg·L^{-1})	0.000[a] (−0.001, 0.000)	**0.029**	−0.192 (−0.259, −0.124)	**<0.001**	0.000 (−0.002, 0.003)	0.735	−0.084 (−0.136, −0.033)	**0.001**	−0.019 (−0.081, 0.044)	0.557	−0.002 (−0.003, −0.001)	**0.002**	0.002 (−0.008, 0.011)	0.724	0.002 (0.001, 0.003)	**0.002**
sTfR (nmol·L^{-1})	0.002 (−0.001, 0.004)	0.212	0.613 (0.117, 1.109)	**0.016**	0.007 (−0.009, 0.022)	0.395	0.523 (0.153, 0.894)	**0.006**	0.356 (−0.086, 0.798)	0.114	0.010 (0.001, 0.019)	**0.025**	0.008 (−0.057, 0.074)	0.803	−0.007 (−0.016, 0.001)	0.100
Haemoglobin (g·dL^{-1})	−0.009 (−0.034, 0.016)	0.468	−2.620 (−8.398, 3.157)	0.373	−0.119 (−0.288, 0.049)	0.165	0.780 (−3.122, 4.682)	0.694	−5.875 (−11.112, −0.637)	**0.028**	0.001 (−0.101, 0.102)	0.991	0.016 (−0.744, 0.776)	0.968	−0.054 (−0.150, 0.041)	0.262
MCV (fL)	−0.007 (−0.013, 0.000)	0.039	−1.206 (−2.715, 0.302)	0.117	0.001 (−0.042, 0.044)	0.963	0.382 (−0.629, 1.394)	0.457	−0.451 (−1.850, 0.947)	0.526	0.005 (−0.021, 0.032)	0.692	0.004 (−0.196, 0.204)	0.967	−0.009 (−0.034, 0.016)	0.472
RDW (%)	0.005 (−0.027, 0.037)	0.767	3.901 (−3.441, 11.242)	0.296	−0.044 (−0.254, 0.167)	0.683	3.799 (−1.091, 8.689)	0.127	9.916 (3.243, 16.589)	**0.004**	0.061 (−0.068, 0.189)	0.353	0.398 (−0.570, 1.365)	0.419	0.012 (−0.109, 0.132)	0.850

1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; βCTX, c-telopeptide cross-links of type 1 collagen; MCV, mean corpuscular volume; PINP, procollagen type 1 N-terminal propeptide; PTH, parathyroid hormone; RDW, erythrocyte distribution width; sTfR, soluble transferrin receptor; 25(OH)D, total 25-hydroxyvitamin D; 24,25(OH)2D, total 24,25-dihydroxyvitamin D.

*controlling for age, height, lean body mass, and habitual exercise volume.

a, coefficient = −0.004

4. Discussion

This study showed that higher ferritin was associated with greater density, size, and strength of the tibia, and lower circulating concentrations of markers of bone resorption and formation in healthy young men. These data provide new insight into associations between iron status and skeletal outcomes measured by HRpQCT. The participants in this study were starting infantry training, one of the British Army's most arduous courses with a high risk of tibial stress fracture [20,21]. Military training diminishes iron status in men and women [10,22–28] and so the data in this study have important implications for managing the skeletal health of military recruits and provide insight into associations between iron status markers and bone structure and metabolism in young men.

4.1 Iron Status and Bone Structure

Higher ferritin was associated with greater density and size of the trabecular (vBMD and volume) and cortical (vBMD, thickness, and area) bone, and higher estimated mechanical strength (stiffness and failure load). Ferritin reflects iron stores in the liver, spleen, and bone marrow [1], but can also be increased by inflammation, acute phase response, and pathologies [40]. The recruits in this study had just completed their initial medical assessment and were declared illness free and ready to train and had yet to complete any military training. Therefore, it is unlikely the ferritin measurements were impacted by illness or exercise-associated inflammation. Ferritin was not associated with bone microstructure (trabecular microarchitecture or cortical porosity) but higher sTfR and RDW—indicators of poorer iron status—were associated with poorer microstructure (lower trabecular number, higher trabecular separation, and higher cortical pore diameter). Haemoglobin had limited associations with tibial structure, likely because most men in this study had normal haemoglobin [11]. Complete depletion of iron stores can occur before haemoglobin is

decreased with low haemoglobin a late phase of iron deficiency [1]. More consistent associations between structural bone outcomes and ferritin might be due to the sensitivity of ferritin to iron stores whereas some measures (*e.g.*, haemoglobin) are only impacted once iron stores are depleted [4] and there was little evidence of poor iron status in the participants in this study. To the author's knowledge, these data provide first evidence of an association between iron status and tibial structure in young men with normal iron status highlighting that iron status may be important for bone structure before levels of deficiency are reached. Despite the low prevalence of iron deficiency, ferritin was low in a high number of men and so an optimal iron status may be important for bone in active young men.

There are limited imaging data exploring iron status and bone structure in humans, but our findings are supported by some DXA studies. Dietary iron intake was positively associated with whole-body, spine, and femur aBMD in postmenopausal women [12]. Data from older adults (> 65 years) from the Korea National Health and Nutrition Examination Survey (KNHANES) demonstrated a positive association between ferritin and aBMD of the lumbar spine and femur in men, but not women [15]. The lack of association in women could be due to the contribution of the menopause to both decreasing aBMD and increasing ferritin due to low oestradiol and the cessation of menstrual bleeding, respectively. In support of this supposition, age and sex stratified analyses from the KNHANES demonstrated negative associations between ferritin and spine and femur aBMD in women over 45 years, but limited association between ferritin and aBMD in younger women [41]. Negative associations between ferritin and femoral neck and / or lumbar spine aBMD have been reported in pre-menopausal women from the KNHANES [42] and US NHANES [43] data. Women can be at risk of iron overload, particularly in the absence of menstrual bleeding (*e.g.*, post-menopause), and iron overload can stimulate osteoclast activity and inhibit osteoblasts function [3,7]. We recently

reported a positive association between ferritin and whole-body aBMD in male and female military recruits, controlling for sex and body size [29]. Animal studies show that restricting dietary iron intake reduces femur and tibial vBMD, cortical thickness and area, and strength [18,19], and decreases lumbar spine trabecular volume and number, and increases trabecular separation [19]. Here we provide evidence for a role of iron status in trabecular and cortical density and size in young men. It is important to confirm these findings in premenopausal women who are at higher risk of iron deficiency and bone stress injuries.

4.2 Iron Status and Bone Metabolism

Higher ferritin was associated with lower βCTX and PINP, measures of type I collagen degradation and formation. Previous studies show higher ferritin was associated with lower circulating concentrations of bone resorption markers [16] and treatment of iron deficiency with iron supplements decreased circulating concentrations of markers of bone resorption and formation [17] in premenopausal women. Higher rates of bone turnover likely contributed to the lower density and size of the trabecular and cortical bone at the ultra-distal tibia in those with lower ferritin [44]. Bone is rich in type I collagen, which is synthesised by the hydroxylation of pro-collagen on proline and lysine residues [6,7]. Hydroxylation is catalysed by prolyl- and lysyl-hydroxylases, which regulate collagen synthesis and are dependent on iron as a cofactor [6,7]. Lower iron stores also cause cell hypoxia, which reduces prolyl hydroxylase activity, increases hypoxia-inducible transcription factor activity, resulting in suppressed osteoclastogenesis and enhanced osteoclast activity [3,4,7]. The lower density and size of the trabecular and cortical bone and higher bone turnover with lower iron stores could be due to reduced collagen synthesis and enhanced osteoclast activity. Lower iron stores could also suppress the hydroxylation of vitamin D by cytochrome P450 enzymes [8] resulting in poorer vitamin D status, lower $1,25(OH)_2D$, and reduced calcium intestinal uptake.

Better iron stores (higher ferritin and lower sTfR) were associated with lower total 25(OH)D (and total 24,25$(OH)_2$D); higher haemoglobin and lower RDW were also associated with lower 1,25$(OH)_2$D. Although there is some evidence for a high prevalence of vitamin D deficiency in iron deficiency [45–47], there is no consistent evidence that increasing iron stores with iron supplementation changes vitamin D status [46], and the direction of the relationships between vitamin D metabolites and iron markers are not clear [3]. Few participants met the World Health Organization definition for iron deficiency (n = 1, ferritin < 15 $\mu g \cdot dL^{-1}$) and anaemia (n = 4, haemoglobin < 13 $g \cdot dL^{-1}$ [11]. We may not have observed a positive relationship between iron and vitamin D metabolites in this study because participants were not iron deficient. Higher total 25(OH)D is associated with reduced inflammation and decreased hepcidin [48,49], which may decrease ferritin and explain the negative relationship between ferritin and total 25(OH)D. Higher ferritin was also associated with higher 1,25$(OH)_2$D:24,25$(OH)_2$D ratio, suggesting the production of 1,25$(OH)_2$D is preferred over 24,25$(OH)_2$D with better iron stores [38]. This association could be consistent with the role of iron in hydroxylation of 25(OH)D to 1,25$(OH)_2$D [8], however, ferritin was also associated with lower total 25(OH)D; lower total 25(OH)D can lead to an increase in 1,25$(OH)_2$D:24,25$(OH)_2$D [38]. Iron status can impact bone through effects of iron on collagen synthesis and vitamin D metabolism. Since we did not observe a consistent positive association between iron status and vitamin D metabolites, we propose the association between iron status and bone in this population of young healthy men is likely due to direct effects of iron on collagen synthesis.

4.3 Limitations

This study did not measure circulating concentrations of hepcidin or transferrin, which could have helped explain some of our findings. Our blood measures were also taken at different times of the day due to the large sample size and limited access time to military recruits. We did not have a measure of dietary iron or calcium intake and it is not clear if other dietary behaviours associated with higher iron intake or serum ferritin contributed to the association with bone outcomes. We also did not have serum measures of other micronutrients or metal elements (including zinc, copper, and lead) that could influence iron status and bone health. We did not include women in this study as no women completed infantry training at the time of data collection; future work should explore associations between iron status and bone microstructure in young women. Our population also had a low prevalence of iron deficiency and future work should explore those with iron deficiency. We did not correct our analysis for the multiple HRpQCT, bone metabolic, and vitamin D metabolite outcomes, and our data should be interpreted considering the chance of type I error. 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. Finally, our data are observational and cannot establish direct causation; future work should seek to directly manipulate iron status and investigate skeletal outcomes.

5. Conclusions

Better iron stores (higher ferritin) were associated with greater density, size, and strength of the tibia, and lower circulating concentrations of markers of bone resorption and formation in young men. The mechanism responsible is likely through the direct role of iron in collagen synthesis.

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Author Contributions

SJ, RMI, NPW, and JPG designed the study. TJO, SJ, NPW, ATC, and SJO 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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