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1 **Long-term follow-up after ovariectomy reveals**
2 **correlations between bone marrow adiposity and**
3 **trabecular bone quality in the proximal metaphysis**
4 **of tibiae in rats**

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20 **Running title:** Adiposity & Bone in Ovariectomized Rats

21 **Abstract**

22 This study aimed to evaluate the correlation between BMAT and bone quality, describe the long-term effects of
23 ovariectomy on bone, and investigate BMAT's spatial distribution. Fifteen-months-old female Sprague–Dawley
24 rats were studied, comparing ovariectomized (OVX, n = 22) and sham-operated (SHAM, n = 11) groups at 6
25 months. Tibias were analyzed for bone microarchitecture, BMAT (microcomputed tomography), mineral
26 parameters (quantitative backscattered electron imaging), and bone composition (Raman microspectroscopy).
27 The OVX tibias showed severe trabecular bone loss (lower bone volume/total volume, $p < 0.001$) with increased
28 BMAT (higher adipose volume per marrow volume, $p < 0.001$), decreased mineral content (lower calcium
29 concentration, $p < 0.001$), and altered organic components (lower mineral/matrix ratio in new bone, $p = 0.03$
30 trabecular surface, $p < 0.001$ trabecular core). When the data are pooled over both groups (SHAM and OVX),
31 the adipose volume/marrow volume ratio was negatively correlated with bone volume/total volume ($r = -0.79$, p
32 < 0.001) and mineral/matrix ratio ($r = -0.37$, $p = 0.04$ trabecular surface; $r = -0.65$, $p < 0.001$ trabecular core)
33 and positively correlated with crystallinity ($r = 0.55$, $p = 0.001$ trabecular surface; $r = 0.49$, $p = 0.006$ trabecular
34 core). The mineral/matrix ratio of trabecular surface new bone was strongly negatively correlated with the
35 adipose compartment nearest to the bone surface. These findings suggest mechanisms underlying BMAT's role
36 in bone resorption.

37 **Keywords**

38 Bone marrow adiposity, Bone, Bone microarchitecture, μ CT, Bone mineral density distribution, Bone
39 ultrastructure, Scanning electron microscopy, Raman microspectroscopy, Rat model

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52 **Formal analysis:** Hélène Béhal (equal), Émeline Cailliau (equal); **Investigation:** Maxime Bedez (lead), Xavier

53 Coutel (support), Alexandre Guille (support); **Resources:** Xavier Coutel; **Writing – Original Draft:** Maxime

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56 Maxime Bedez (lead), Thomas Fasquelle (support); **Funding acquisition:** Xavier Coutel.

57 **Abbreviations**

58 μ CT: Microcomputed Tomography

59 AdV/MaV: Adipose Volume to Marrow Volume Ratio

60 BMAd: Bone Marrow Adipocyte

61 BMAT: Bone Marrow Adipose Tissue

62 BMD: Bone Mineral Density

63 BMDD: Bone Mineralization Density Distribution

64 BSE image: Backscattered Electron image

65 BV/TV: Bone Volume to Total Volume Ratio

66 Ca_{Mean} : Average Calcium Concentration

67 Ca_{Peak} : Most Frequently Occurring Calcium Concentration

68 CARB: Relative Carbonate B Content

69 Ca_{Width} : Full-Width at Half Maximum of the Calcium Distribution

70 COLL: Collagen Maturity

71 CRYST: Mineral Crystals' Crystallinity

72 ESEM: Environmental Scanning Electron Microscopy

73 F_{Peak} : Bone Surface Frequency of the Most Frequently Occurring Calcium Concentration

74 HPP: Hydroxyproline/Proline Ratio

75 MgF_2 : Magnesium Fluoride

- 76 MMR: Mineral-to-Matrix Ratio
- 77 NBF: Neutral Buffered Formalin
- 78 NB-Tb.C: New Bone, Trabecular Core
- 79 NB-Tb.S: New Bone, Trabecular Surface
- 80 OB-Tb.C: Old Bone, Trabecular Core
- 81 OB-Tb.S: Old Bone, Trabecular Surface
- 82 OVX: Ovariectomized group
- 83 PBS: Phosphate-Buffered Saline
- 84 GAGs: Glycosaminoglycans
- 85 PMMA: polymethylmethacrylate
- 86 qBEI: Quantitative Backscattered Electron Imaging
- 87 SHAM: Sham-operated group
- 88 vBMD: Volumetric Bone Mineral Density
- 89 β -TCP: Tricalcium Phosphate

90 **Introduction**

91 Osteoporosis is marked by an imbalance in bone remodeling due to estrogen deficiency during menopause,
92 leading to changes in bone quality [1]. Fracture risk depends on both bone quantity and quality. Bone loss
93 mainly affects trabecular bone and increases bone marrow adipose tissue (BMAT) volume in specific skeletal
94 sites. There is a well-established negative correlation between BMAT and bone quality in settings of
95 osteoporosis [2] that has historically been explained by two hypotheses: a shift in progenitor differentiation
96 favoring adipocytes over osteoblasts or adipocytes regulating the microenvironment to promote bone resorption
97 [3]. As correlation does not necessarily equate to causation, emerging literature also reveals that bone loss in
98 settings of ovariectomy, diabetes, and with age can occur independent of BMAT expansion [4-6]. This remains
99 an important point to clarify as inhibiting fatty acids in vitro prevents adipocyte lipotoxicity in osteoblasts [7].
100 Previous research also indicates that BMAT in the proximal tibia is closer to trabecular bone than in the
101 mandible, clustering even closer during osteoporosis like conditions [8,9]. Our hypothesis is that BMAT
102 undergoes quantitative expansion and spatial distribution changes in osteoporosis.
103 Osteoporotic bone shows lower calcium content and specific bone mineralization profiles [10]. Environmental
104 scanning electron microscopy (ESEM) can differentiate between newly formed and older, mature bone based on
105 calcium content [11]. Bone strength is affected by bone quantity, ultrastructural composition, and calcium
106 density distribution [12]. Raman microspectroscopy precisely analyzes mineral and organic components,
107 distinguishing new from old bone [13,14].
108 This study explores both normal and osteoporotic bone through relative tissue age extrapolated by equivalent
109 calcium content and BMAT spatial distribution, an area previously understudied. We assessed the role of BMAT
110 distribution in bone microarchitecture, mineral parameters, and composition in new and old bone. Using a
111 surgical model of osteoporosis induced by ovariectomy in female rats, we aimed to evaluate the relationship
112 between BMAT and bone quality under both normal and osteoporosis conditions. Secondary objectives included
113 examining the long-term effects of ovariectomy on bone microarchitecture, mineral parameters, and
114 composition, and investigating these parameters across three distinct BMAT spatial compartments.

115 **Material and methods**

116 **1. Experimental rat model and sample preparation**

117 The guidelines for reporting animal research were followed (ARRIVE 2.0). The protocol (APAFIS#4197-
118 2015091514435707) was approved by the National Committee on Ethics in Animal Experimentation (CEEA
119 075) and legal requirements in France for the care and use of animals were followed. Thirty-three Sprague–
120 Dawley rats (*Rattus norvegicus*) aged six months and with homogeneous weights at baseline (Janvier Lab,
121 Laval, France) [8] were studied. The rats were housed at the animal facility at the University of Lille (DHURE).
122 The rats (n = 3 per cage) were housed in type 4 cages filled with Lignocel™ bedding and provided with
123 horizontal tubes for climbing under controlled conditions at 22 ± 2 °C on a 12-hour light/12-hour dark cycle.
124 Each animal was monitored daily and observed to determine any signs of poor adaptation to its environment.
125 Assignments and surgeries were performed at 6 months by the provider to constitute a sham surgery group
126 (SHAM, n = 11) and an ovariectomy group (OVX, n = 22). The assignment of the rats to groups was random.
127 All experimenters were aware of the group allocation during performance of the experiment, result evaluation,
128 and data analysis. The rats were sacrificed by exsanguination under anesthesia at 15 months of age (9 months
129 post-OVX).
130 We previously performed and published our method for the collection and preparation of the tibia samples [8].
131 The right tibias were subjected to analysis of the bone microarchitecture using microcomputed tomography
132 (μ CT). The tibias were harvested, fixed for 48 hours in 10% neutral buffered formalin (NBF), and then stored in
133 phosphate-buffered saline (PBS) for a first μ CT acquisition. Subsequently, they were prepared for analysis of
134 BMAT via decalcification, osmium tetroxide staining and storage in PBS, for a second μ CT acquisition. The left
135 tibias were used for the analysis of mineral parameters through quantitative backscattered electron imaging
136 (qBEI) and for the analysis of bone composition through Raman microspectroscopy. The left tibias were fixed in
137 70% ethanol for 48 hours before being embedded in polymethylmethacrylate (PMMA) resin. They were then
138 sectioned and polished to a thickness of 100 μ m.

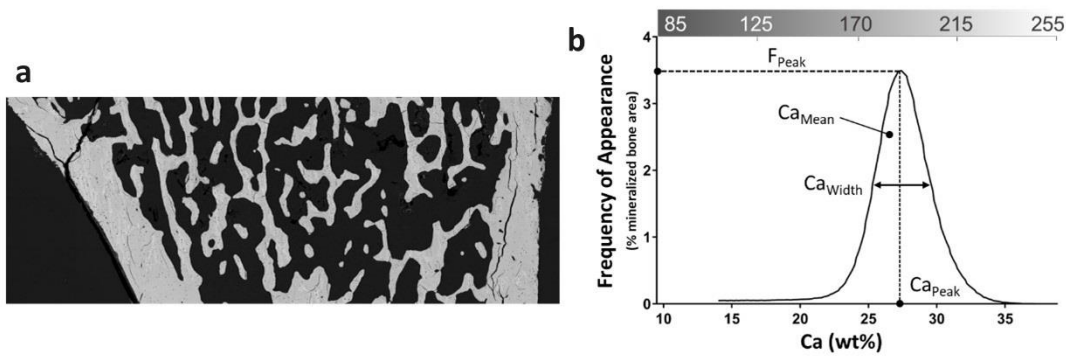
139 **2. X-ray microcomputed tomography**

140 The data and analysis protocol for bone microarchitecture and BMAT content investigation were previously
141 published by our laboratory team [8]. The initial acquisitions were performed using a Skyscan 1172 μ CT device
142 (Bruker MicroCT, Kontich, Belgium) with the following parameters: isotropic voxels of $10 \mu\text{m}^3$, 80 kVp,
143 100 μ A, an Al-Cu filter, an integration time of 2400 ms and a rotation step of 0.5° over 180° . The data

144 acquisition, reconstruction, analysis, and three-dimensional visualization were conducted using the following
145 software: Nrecon™, Dataviewer™, CTAn™, and CTVox™ (Bruker μ CT, Kontich, Belgium). Each bone
146 sample was assessed before and after decalcification and osmium tetroxide staining. Osmium tetroxide binds to
147 lipids stored in the cytoplasm of bone marrow adipocytes (BMAds), revealing BMAT in μ CT. The region of
148 interest is a cross-sectional slice of 2 mm thickness, located 1.5 mm below the growth plate. This protocol was
149 carried out on the 33 tibias in this study. Bone density was represented by the ratio of bone volume to total
150 volume (BV/TV, expressed as a percentage). BMAT was represented by the ratio of adipose volume to marrow
151 volume (AdV/MaV, expressed as a percentage). The bone marrow was divided into 20 μ m compartments
152 relative to the surface of the trabecular bone (D1: 0-20 μ m, D2: 20-40 μ m, D3: 40-60 μ m) to study the spatial
153 distribution of BMAT.

154 **3. Environmental scanning electron microscopy and bone mineral** 155 **density distribution**

156 The PMMA-embedded samples were analyzed using an environmental scanning electron microscope (Quanta
157 200™, FEI, Hillsboro, OR, USA) coupled with an energy-dispersive X-ray spectrometry detector (QuanTax™,
158 Bruker). The parameters used included an accelerating voltage of 20 kV and a working distance of 10 mm. The
159 bone mineral density distribution (BMDD) was evaluated using the method described by Roschger [15] and
160 adapted by Olejnik [16]. The recorded images (Fig. 1a) are backscattered electron (BSE) images. The BSE
161 images are displayed in grayscale. The BSE grayscale was calibrated using the “atomic number (Z) contrast” of
162 reference materials, using PMMA resin ($Z = 6$), aluminium (Al, $Z = 13$), magnesium fluoride (MgF_2 ,
163 $Z_{\text{mean}}=10$) and tricalcium phosphate (β -TCP, $Z_{\text{mean}}=14.4$). The experimental gray-levels of PMMA and β -
164 TCP were taken as 0% and 38.7% of weight of calcium, respectively. The BE gray-level was converted into
165 weight concentration calcium. The intensity of the grayscale levels is dependent on the amount of calcium. The
166 grayscale curve was extracted from the BSE images (Fig. 1b – upper x-axis). This curve corresponded to the
167 BMDD. Through instrument calibration, the grayscale curve was converted into the mass percentage of calcium
168 (Fig. 1b – bottom x-axis). The BMDD depicts the percentage of bone surface as a function of the percentage of
169 calcium. Four parameters were calculated from the BMDD: a) the full width at half maximum (Ca_{Width}),
170 expressed as the mass percentage of calcium; b) the most frequently occurring calcium concentration (Ca_{Peak}),
171 expressed as the weight percentage of calcium; c) the bone surface frequency of Ca_{peak} (F_{Peak}), expressed as a
172 percentage; and d) the average calcium concentration (Ca_{Mean}), expressed as the mass percentage of calcium.
173 Calculations were performed using MATLAB software (R2023a; MathWorks, Natick, MA, USA).



174

175 **Fig. 1** Grayscale BSE image of the trabecular and cortical bone in a left tibia from the SHAM group (a). The
 176 grayscale curve ranging from 85 to 255 (upper x-axis), with grayscale levels converted to mass percentages of
 177 calcium ranging from 0 to 38.7% (lower x-axis) (b). This curve is designated the BMDD and is characterized by
 178 the parameters Ca_{width} , Ca_{Peak} , F_{Peak} , and Ca_{Mean} . Only trabecular bones were chosen for the calculation of the
 179 **BMDD**

180 BSE images enable the localization of bone surface areas based on their calcium content. The images were
 181 segmented into three natures of packets: PMMA resin (black), newly formed bone (new bone), and mature bone
 182 (old bone). New and old bone packets were differentiated according to the calcium content: new bone was
 183 represented by a calcium content ranging from 0% to 90% of the maximum (primary mineralization), while old
 184 bone was represented by a calcium content ranging from 90% to 100% (secondary mineralization) [17].
 185 Standardizing the areas of new bone and old bone based on calcium content allows for the identification of
 186 relevant analysis regions for Raman microspectroscopy measurements. Only BSE images containing sufficient
 187 trabecular bone were used for this analysis. Cortical bone was not considered in this study.

188 **4. Raman microspectroscopy**

189 Raman analyses were conducted using a LabRAM HR800 microspectrometer (HORIBA, Jobin Yvon,
 190 Villeneuve d'Ascq, France) equipped with a diode laser (785 nm, 100 mW), a CCD detector (1024 by 256
 191 pixels), and a $\times 100$ objective (NA = 0.80; Olympus, France). The spectral acquisition window was set between
 192 300 and 1800 cm^{-1} . The lateral resolution was 1 μm . A scrambler was used to minimize polarization effects. The
 193 acquisition time consisted of two acquisitions of 30 seconds each. Four bone areas were identified from the BSE
 194 images (see Fig. 2): new bone – trabecular surface (NB-Tb.S), new bone – trabecular core (NB-Tb.C), old bone
 195 – trabecular surface (OB-Tb.S), and old bone – trabecular core (OB-Tb.C). For each sample and each bone area,
 196 ten Raman spectra were acquired. All Raman spectra were processed using LabSpec 6 software (HORIBA, Jobin
 197 Yvon, France). The physicochemical parameters were calculated using MATLAB software (R2023a;
 198 MathWorks, USA) as follows.

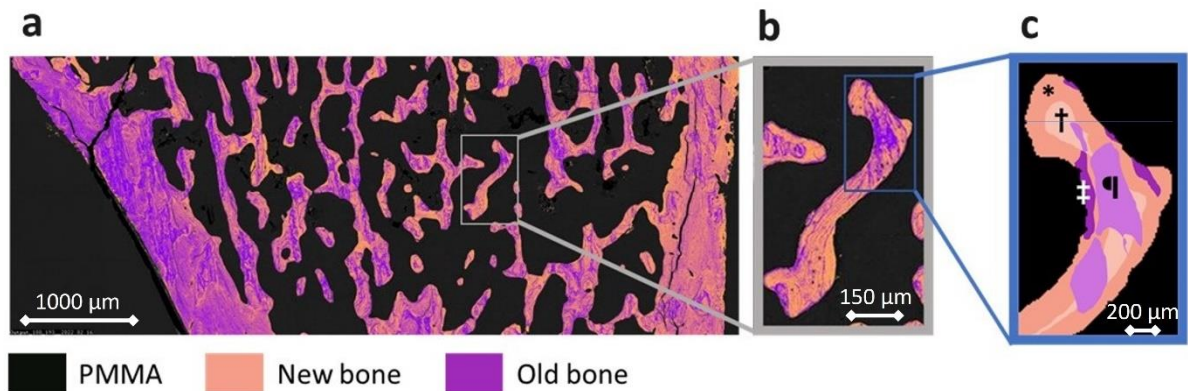


Fig. 2 The grayscale BSE image was colored into black (grayscale 0-99), pink (grayscale 100-193, accounting for 90% of mineralization) and purple (grayscale 194-255) to locate new bone and old bone prior to the area selection on the Raman microspectrometer (a). A magnified view of a trabecula (b). Simplified image of the four categories, showing position (surface or core) and mineralization (new bone or old bone) (c). * new bone – trabecular surface; † new bone – trabecular core; ‡ old bone, trabecular surface; ¶ old bone, trabecular core

The mineral-to-matrix ratio (MMR) describes the amount of mineral relative to the amount of organic matrix in bone [13]. It is the ratio of the area under the $\nu_1\text{PO}_4$ peak (range 900-990 cm^{-1}) to that of the $\delta(\text{CH}_2)$ peak of collagen (range 1434-1490 cm^{-1}). Crystallinity (CRYST) refers to the size and perfection of mineral crystals, which gradually increase with the formation of apatite [13]. This peak is the inverse of the full width at half maximum of the $\nu_1\text{PO}_4$ peak. The relative content of carbonate B (CARB) measures the amount of type B carbonates in the bone mineral [13]. It is the ratio of the area under the CO_3^{2-} type B peak (range 1052-1092 cm^{-1}) to that of the $\nu_1\text{PO}_4$ peak. The hydroxyproline/proline ratio (HPP) provides information about the posttranslational modifications of collagen [18]. It is calculated by the ratio of the intensity of the proline peak (range 828-898 cm^{-1}) to that of the hydroxyproline peak (range 828-898 cm^{-1}). Collagen maturity (COLL) corresponds to collagen crosslinking modifications [13,19]. It is calculated by the ratio of the intensity between two successive amide I peaks (1660 cm^{-1} and 1690 cm^{-1}). The relative content of glycosaminoglycans (GAGs) is indicative of noncollagenous organic constituents [13]. Glycosaminoglycans play a role in assembling the organic content, modulating mineralization and remodeling, and preserving the nonmineralized organic matrix of osteocytes and canaliculi. It is calculated by the ratio of the area under the glycosaminoglycan peak (range 1365-1390 cm^{-1}) to that of the amide III peak (range 1243-1269 cm^{-1}). These six parameters were measured at four different bone locations: NB-Tb.S, OB-Tb.C, NB-Tb.C, and OB-Tb.S (Fig. 2).

221 **5. Statistical analysis**

222 Qualitative variables are described in terms of frequencies and percentages. Quantitative variables are described
223 in terms of the mean and standard deviation. The normality of distributions was assessed visually and using the
224 Shapiro–Wilk test.

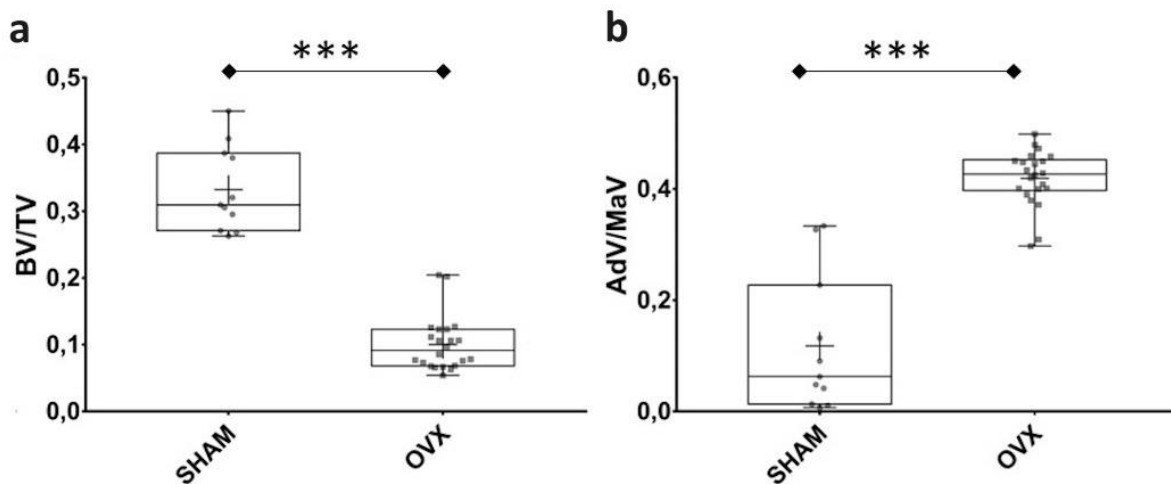
225 Comparisons between two elements were conducted using the Student’s t test for normally distributed data and
226 the Mann–Whitney test for nonnormally distributed data. Multiple comparisons were performed using one-way
227 ANOVA for normally distributed data and the Kruskal–Wallis test for nonnormally distributed data. When a
228 significant difference was found, a post hoc Dunn’s test was applied to characterize the difference. The
229 relationship between the parameters and adiposity was assessed using the Pearson correlation coefficient and its
230 associated test. Each correlation was assessed for the whole sample and for separate groups. For parameters
231 significantly associated with adiposity at a threshold of 0.10, we investigated, using the same method, the
232 relationship between the parameter and adiposity in each of the three compartments (D1, D2, and D3). Statistical
233 comparison analyses were conducted using GraphPad Prism v7.0 software (GraphPad Software, San Diego, CA,
234 USA). Statistical correlation analyses were performed using SAS software (SAS Institute version 9.4). The
235 significance level was set at 5%.

236 **Results**

237 **1. Effects of ovariectomy on trabecular bone**

238 **a. Bone loss and BMAT gain**

239 The BV/TV and the AdV/MaV within the trabecular bone were compared between the SHAM and OVX groups.
240 The OVX group exhibited a significantly lower BV/TV ratio than did the SHAM group (median = 11.1% [Q1 =
241 7.5%, Q3 = 12.4%] vs. median = 31.0% [Q1 = 28.3%, Q3 = 38.6%], $p < 0.001$ – Fig. 3a). The OVX group
242 exhibited a significantly greater AdV/MaV ratio than did the SHAM group (median = 42.7% [Q1 = 36.7%, Q3 =
243 48.3%] vs. median = 7.5% [Q1 = 1.2%, Q3 = 23.1%], $p < 0.001$ – Fig. 3b).

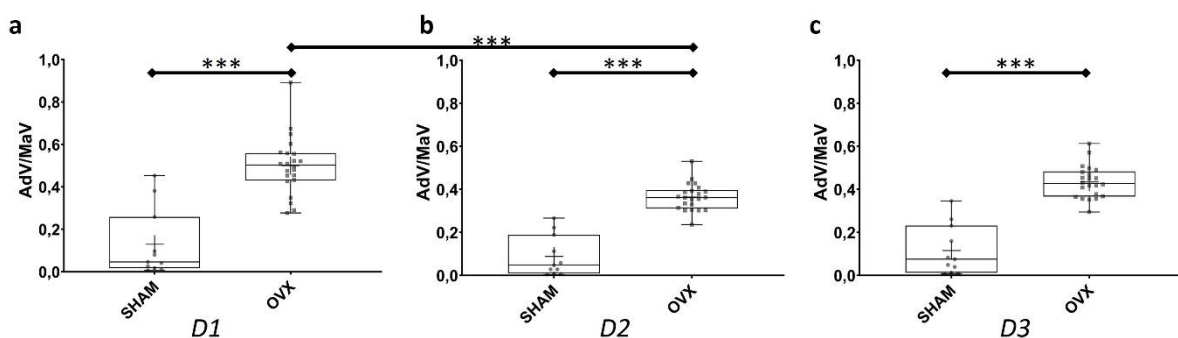


244

245 **Fig. 3** Bone volume / Total Volume (BV/TV) ratios of the SHAM and OVX groups in trabecular bone of the
 246 tibiae. The region of interest is a cross-sectional slice of 2 mm thickness, located 1.5 mm below the growth plate.

247 (a). The adipose volume to marrow volume (AdV/MaV) ratios of the SHAM and OVX groups (b). The cross
 248 represents the mean. ***: $p < 0.001$

249 The AdV/MaV parameter was assessed based on its proximity to the trabecular bone surface. The bone marrow
 250 adjacent to the bone surface was segmented into three compartments according to their distance from the
 251 surface: D1 (0-20 μm), D2 (20-40 μm), and D3 (40-60 μm). The difference in the AdV/MaV ratios between the
 252 SHAM and OVX groups remained significant in compartments D1, D2, and D3 ($p < 0.001$ for all three – Fig. 4).
 253 The parameter AdV/MaV did not differ across the three compartments in the SHAM group (D1-D2: $p = 0.73$;
 254 D2-D3: $p = 0.87$; D1-D3: $p = 0.97$ – Fig. 4). In the OVX group, the AdV/MaV ratio was greater at D1 than at D2
 255 ($p < 0.001$). The other compartments did not show differences (D2-D3: $p = 0.05$; D1-D3: $p = 0.07$).



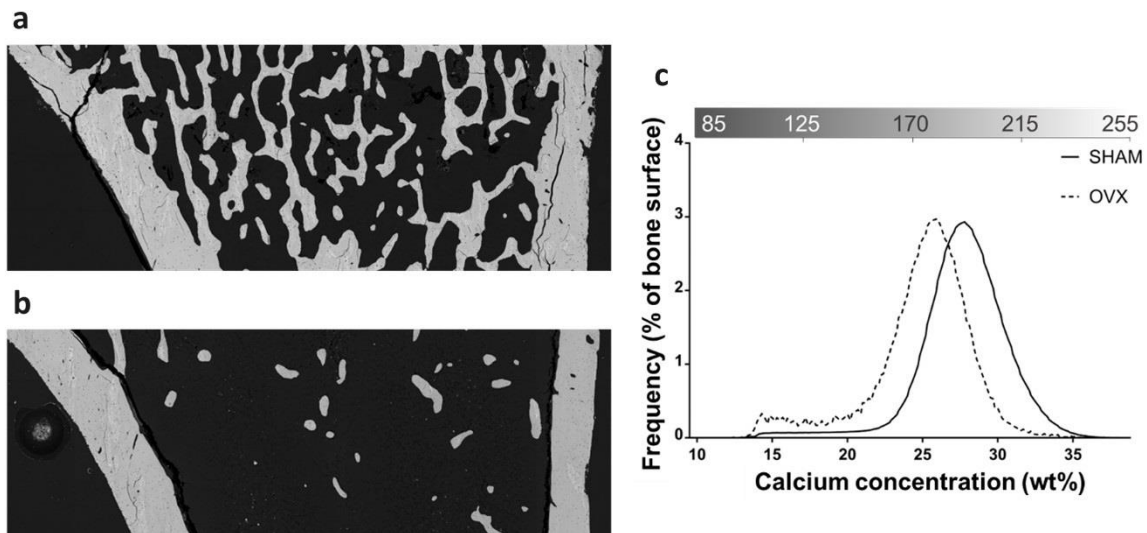
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257 **Fig. 4** Adipose volume to total volume (AdV/MaV) ratios of the SHAM and OVX groups based on spatial
 258 distribution. (A) D1: 0-20 μm ; (B) D2: 20-40 μm ; (C) D3: 40-60 μm . The cross represents the mean.

259 ***: $p < 0.001$

b. Bone mineral density distribution (BMDD)

260
261 The trabecular bones of the SHAM and OVX groups were analyzed using ESEM imaging and qBEI. Fig. 5a and
262 5b show a representative calcium content representation on BSE images of the trabecular bones of the SHAM
263 and OVX groups, respectively. The SHAM trabecular bones exhibited quantitative modifications, with a higher
264 number of trabeculae compared to those in the OVX group. The trabecular BMDD of the OVX group showed a
265 significant shift toward a low concentration of Ca compared to the BMDD of the SHAM group (Fig. 5c,
266 Table 1). The Ca_{width} of the OVX group was significantly lower compared to the Ca_{width} of the SHAM group
267 (median = 4.01 [Q1 = 3.89, Q3 = 4.27] vs. median = 4.56 [Q1 = 4.28, Q3 = 5.03], $p < 0.001$). The Ca_{Mean} of the
268 OVX group was significantly lower compared to the Ca_{Mean} of the SHAM group (median = 25.33% [Q1 =
269 24.90%, Q3 = 25.66% vs. median = 27.59% [Q1 = 27.24%, Q3 = 28.60], $p < 0.001$).



270
271 **Fig. 5** Backscattered electron (BSE) image of a proximal metaphysis of a tibia from the SHAM group (a). BSE
272 image of a proximal metaphysis of a tibia from the OVX group (b). BMDD of the SHAM (solid lines) and OVX
273 (dash lines) groups (c)

274 One left tibia sample did not contain trabecular bone; the corresponding mineral and bone composition
275 parameters were not evaluated.

c. Raman microspectroscopy

276
277 Raman spectra were acquired in bone packets depending on bone age. The bone packets were identified in BSE
278 images and labeled as follows: NB-Tb.C, NB-Tb.S, OB-Tb.C, and OB-Tb.S. The physico-chemical parameters
279 were calculated and compared between the SHAM and OVX groups depending on the bone age.

280 The OVX group exhibited a lower MMR than the SHAM group in the NB-Tb.S (10.63 ± 1.4 vs. 11.95 ± 1.8 , $p =$
281 0.03) and in the NB-Tb.C (11.71 ± 1.50 vs. 14.23 ± 1.60 , $p < 0.001$). The OVX group exhibited a higher CRYST
282 than the SHAM group in the NB-Tb.S (0.057 ± 0.001 vs. 0.056 ± 0.000 , $p = 0.003$) and the NB-Tb.C
283 (0.057 ± 0.001 vs. 0.056 ± 0.001 , $p < 0.001$). We did not find significant differences in the parameters of bone
284 composition in the old bone (surface or core). No significant difference was found between the OVX and SHAM
285 groups for the following parameters: CARB, COLL, and GAG. Due to the low number of trabeculae in OVX
286 (Figure 5 b), some samples did not have old bone (according Ruffoni description). Thus, some Raman
287 acquisitions could not be performed on old bone in these specific samples.

288 **Table 1** Overview of the parameters measured in the two groups SHAM and OVX

		SHAM		OVX		P
		N	Mean (SD) Median (Q1;Q3)	N	Mean (SD) Median (Q1;Q3)	
μCT						
	BV/TV (%)	11	31.0 (28.3;38.6)	22	11.1 (7.5;12.4)	< 0.001 ^a
	AdV/MaV (%)	11	6.2 (1.3;22.7)	22	42.7 (39.7;45.2)	< 0.001 ^a
	AdV/MaV _{D1} (%)	11	4.6 (1.7;25.8)	22	50.3 (43.1;55.9)	< 0.001 ^a
	AdV/MaV _{D2} (%)	11	4.8 (0.9;18.8)	22	36.2 (31.2;39.7)	< 0.001 ^a
	AdV/MaV _{D3} (%)	11	7.5 (1.2;23.1)	22	42.7 (36.7;48.3)	< 0.001 ^a
BMDD						
	Ca _{width} (wt%)	11	4.56 (4.28;5.03)	21	4.01 (3.89;4.27)	< 0.001 ^a
	Ca _{Peak} (wt%)	11	27.76 (27.41;28.62)	21	25.69 (25.34;26.38)	< 0.001 ^a
	Ca _{Mean} (wt%)	11	27.59 (27.24;28.60)	21	25.33 (24.90;25.66)	< 0.001 ^a
	F _{Peak} (%)	11	3.234 (0.297)	21	3.556 (0.257)	0.003 ^b
Physico-chemical parameters: bone ultrastructural composition (Raman)						
<i>NB-Tb.S</i>	MMR_NB-Tb.S	11	11.95 (1.836)	20	10.63 (1.401)	0.03 ^b
	CARB_NB-Tb.S	11	0.094 (0.008)	20	0.097 (0.008)	0.46 ^b
	CRYST_NB-Tb.S	11	0.056 (0.001)	20	0.057 (0.001)	0.003 ^b
	COLL_NB-Tb.S	11	1.427 (0.087)	20	1.534 (0.182)	0.08 ^b
	HPP_NB-Tb.S	11	0.530 (0.480;0.570)	20	0.540 (0.503;0.603)	0.36 ^a
	GAG_NB-Tb.S	11	0.063 (0.022)	20	0.053 (0.013)	0.11 ^b
<i>OB-Tb.C</i>	MMR_OB-Tb.C	11	16.65 (2.631)	7	14.97 (3.369)	0.25 ^b
	CARB_OB-Tb.C	11	0.103 (0.012)	7	0.107 (0.014)	0.56 ^b
	CRYST_OB-Tb.C	11	0.056 (0.001)	7	0.056 (0.000)	0.61 ^b
	COLL_OB-Tb.C	11	1.380 (0.159)	7	1.479 (0.079)	0.15 ^b
	HPP_OB-Tb.C	11	0.419 (0.067)	7	0.477 (0.091)	0.14 ^b
	GAG_OB-Tb.C	11	0.080 (0.029)	7	0.085 (0.034)	0.75 ^b
<i>NB-Tb.C</i>	MMR_NB-Tb.C	11	14.23 (1.602)	19	11.71 (1.497)	< 0.001 ^b
	CARB_NB-Tb.C	11	0.102 (0.005)	19	0.106 (0.007)	0.13 ^b
	CRYST_NB-Tb.C	11	0.056 (0.000)	19	0.057 (0.001)	< 0.001 ^b
	COLL_NB-Tb.C	11	1.488 (0.093)	19	1.434 (0.108)	0.18 ^b
	HPP_NB-Tb.C	11	0.496 (0.056)	19	0.552 (0.073)	0.04 ^b
	GAG_NB-Tb.C	11	0.053 (0.019)	18	0.057 (0.011)	0.45 ^b
<i>OB-Tb.S</i>	MMR_OB-Tb.S	11	18.30 (4.088)	3	12.68 (4.364)	NA
	CARB_OB-Tb.S	11	0.109 (0.010)	3	0.114 (0.014)	NA
	CRYST_OB-Tb.S	11	0.056 (0.001)	3	0.057 (0.000)	NA
	COLL_OB-Tb.S	11	1.613 (0.246)	3	1.474 (0.122)	NA
	HPP_OB-Tb.S	11	0.401 (0.078)	3	0.410 (0.044)	NA
	GAG_OB-Tb.S	8	0.075 (0.026)	3	0.063 (0.035)	NA

289 *BMAT*: bone marrow adipose tissue, *μCT*: microcomputed tomography; *BMDD*: bone mineral density

290 distribution; *AdV/MaV*: adipose volume to marrow volume ratio; *BV/TV*: bone volume to total volume ratio;

291 *Ca_{width}*: full-width at half maximum of the calcium distribution; *Ca_{Peak}*: most frequently occurring calcium

292 concentration; *F_{Peak}*: bone surface frequency of the most frequently occurring calcium concentration; *Ca_{Mean}*:

293 average calcium concentration; *MMR*: mineral-to-matrix ratio; *CARB*: relative carbonate B content; *CRYST*:

294 mineral crystals' crystallinity; *COLL*: collagen maturity; *HPP*: hydroxyproline/proline ratio; *GAG*:

295 glycosaminoglycans; *NB-Tb.S*: new bone – trabecular surface; *OB-Tb.C*: old bone – trabecular core; *NB-Tb.C*:

296 new bone – trabecular core; *OB-Tb.S*: old bone – trabecular surface; *N*: sample size; ^aMann–Whitney test;

297 ^bStudent's test; *NA*: not applicable; Bold indicates significant differences at the *p* = 0.05 threshold

298 **2. Correlations with BMAT**

299 Correlations were investigated to explore potential associations between BMAT and bone quality. Correlations
300 were assessed for pooled data (SHAM and OVX) and for each group. The correlations between BMAT and bone
301 loss, mineral parameters, and bone composition are provided in Table 2.

302 When data are pooled, a strong negative correlation was observed between the AdV/MaV ratio and the BV/TV
303 ratio ($r = -0.79$, $p < 0.001$). Strong correlations between the AdV/MaV ratio and mineral parameters were also
304 observed ($r = -0.59$, $p < 0.001$ Ca_{Width}; $r = -0.67$, $p < 0.001$ with Ca_{Peak}; $r = 0.48$, $p = 0.005$; and $r = -0.75$,
305 $p < 0.001$ with Ca_{Mean}). Three strong correlations between the AdV/MaV ratio and Raman parameters were
306 observed: CRYST_NB-Tb.S, MMR_NB-Tb.C, and CRYST_NB-Tb.C ($r = 0.55$, $p = 0.001$; $r = -0.65$, $p < 0.001$;
307 and $r = 0.49$, $p = 0.006$, respectively).

308 The correlation between the AdV/MaV ratio and the BV/TV ratio is not significant in SHAM, and is strong and
309 negative in OVX ($r = -0.54$, $p = 0.009$). The correlation between the AdV/MaV ratio and COLL_NB-Tb.C is
310 strong and positive in SHAM ($r = 0.63$, $p = 0.03$), and strong and negative in OVX ($r = -0.69$, $p < 0.001$).

311 The analysis did not reveal a correlation between the AdV/MaV ratio and CARB or GAG (across all zones). In
312 the old bone – trabecular cores, no correlations were observed either.

Table 2 Correlations of the AdV/MaV ratios of the whole sample and separate groups.

	Whole sample			SHAM			OVX			
	N	r	p	N	r	p	N	r	p	
Correlation with bone density										
\$ BV/TV	33	-0.79	< 0.001	11	0.41	0.21	22	-0.54	0.009	
Correlations with bone mineral density distribution										
\$ Ca _{width}	32	-0.59	< 0.001	11	-0.06	0.87	21	-0.27	0.24	
\$ Ca _{Peak}	32	-0.67	< 0.001	11	-0.16	0.65	21	-0.10	0.67	
\$ F _{Peak}	32	0.48	0.005	11	0.04	0.91	21	0.19	0.41	
\$ Ca _{Mean}	32	-0.75	< 0.001	11	-0.20	0.57	21	-0.22	0.35	
Correlations with Raman microspectroscopy										
NB-Tb.S	\$ MMR_NB-Tb.S	31	-0.37	0.04	11	-0.15	0.67	20	0.05	0.85
	\$ CARB_NB-Tb.S	31	0.04	0.85	11	-0.30	0.39	20	-0.05	0.85
	\$ CRYST_NB-Tb.S	31	0.55	0.001	11	0.38	0.26	20	0.17	0.49
	\$ COLL_NB-Tb.S	31	0.26	0.15	11	-0.02	0.96	20	-0.06	0.82
	\$ HPP_NB-Tb.S	31	-0.01	0.94	11	-0.10	0.79	20	0.01	0.98
	\$ GAG_NB-Tb.S	31	-0.20	0.28	11	0.10	0.77	20	0.18	0.46
OB-Tb.C	\$ MMR_OB-Tb.C	18	-0.22	0.39	11	0.19	0.59	7	-0.41	0.38
	\$ CARB_OB-Tb.C	18	0.10	0.69	11	0.01	0.97	7	-0.15	0.76
	\$ CRYST_OB-Tb.C	18	0.21	0.41	11	0.15	0.67	7	0.35	0.46
	\$ COLL_OB-Tb.C	18	0.09	0.74	11	-0.34	0.32	7	-0.48	0.30
	\$ HPP_OB-Tb.C	18	0.35	0.15	11	0.02	0.96	7	0.37	0.44
	\$ GAG_OB-Tb.C	18	0.13	0.60	11	0.31	0.37	7	-0.36	0.45
NB-Tb.C	\$ MMR_NB-Tb.C	30	-0.65	< 0.001	11	-0.33	0.33	19	-0.22	0.38
	\$ CARB_NB-Tb.C	30	0.20	0.29	11	0.07	0.84	19	-0.28	0.26
	\$ CRYST_NB-Tb.C*	30	0.49	0.006	11	-0.33	0.34	19	0.08	0.76
	\$ COLL_NB-Tb.C	30	-0.22	0.24	11	0.63	0.03	19	-0.69	< 0.001
	\$ HPP_NB-Tb.C	30	0.39	0.03	11	0.01	0.98	19	0.30	0.21
	\$ GAG_NB-Tb.C	29	0.17	0.39	11	0.18	0.60	18	-0.16	0.54
OB-Tb.S	\$ MMR_OB-Tb.S	14	-0.31	0.28	11	0.01	0.98	3	NA	NA
	\$ CARB_OB-Tb.S	14	0.31	0.30	11	0.10	0.78	3	NA	NA
	\$ CRYST_OB-Tb.S	14	0.18	0.56	11	0.10	0.78	3	NA	NA
	\$ COLL_OB-Tb.S	14	-0.07	0.82	11	0.25	0.48	3	NA	NA
	\$ HPP_OB-Tb.S	14	-0.20	0.49	11	-0.37	0.27	3	NA	NA
	\$ GAG_OB-Tb.S	11	-0.17	0.64	8	-0.06	0.89	3	NA	NA

314 AdV/MaV: adipose volume to marrow volume ratio; BV/TV: bone volume to total volume ratio; Ca_{width}: full-
315 width at half maximum of the calcium distribution; Ca_{Peak}: most frequently occurring calcium concentration;
316 F_{Peak}: bone surface frequency of the most frequently occurring calcium concentration; Ca_{Mean}: average calcium
317 concentration; MMR: mineral-to-matrix ratio; CARB: relative carbonate B content; CRYST: mineral crystals'
318 crystallinity; COLL: collagen maturity; HPP: hydroxyproline/proline ratio; GAG: glycosaminoglycans; NB-
319 Tb.S: new bone – trabecular surface; OB-Tb.C: old bone – trabecular core; NB-Tb.C: new bone – trabecular
320 core; OB-Tb.S: old bone – trabecular surface; N = sample size. The values are expressed as the Pearson
321 correlation coefficient (r) and significance (p). The symbol \$ indicates correlation with the AdV/MaV ratio.
322 Strong correlations (r > 0.50) that are statistically significant at the p = 0.05 threshold are highlighted in bold.
323 *CRYST_NB-Tb.C (r = 0.49) has been included in the strong correlations.

3. Correlation with BMAT based on spatial distribution

In the previous section, eight parameters were significantly correlated ($p < 0.05$) when evaluating the pooled SHAM and OVX groups. These eight parameters were analyzed based on their spatial distribution (D1, D2, and D3). The correlations between BMAT and eight parameters are presented in Table 3 depending on their spatial distribution. The direction of the correlations was consistent across compartments. However, we noticed slight differences according to the spatial distribution for AdV/MaV \$ BV/TV, AdV/MaV \$ F_{Peak} , AdV/MaV \$ MMR_NB-Tb.S and AdV/MaV \$ HPP_NB-Tb.C, but the correlations remain on the same order of magnitude.

Table 3 Pearson correlations of the pooled parameters AdV/MaV_{D1}, AdV/MaV_{D2}, and AdV/MaV_{D3} (SHAM and OVX) based on the spatial distribution of significantly correlated parameters ($p < 0.05$)

	\$ AdV/MaV _{D1}			\$ AdV/MaV _{D2}			\$ AdV/MaV _{D3}		
	N	r	p	N	r	p	N	r	p
BV/TV	33	-0.70	< 0.001	33	-0.81	< 0.001	33	-0.80	< 0.001
Ca _{Width}	32	-0.60	< 0.001	32	-0.61	< 0.001	32	-0.58	< 0.001
Ca _{Peak}	32	-0.70	< 0.001	32	-0.69	< 0.001	32	-0.68	< 0.001
F _{Peak}	32	0.57	< 0.001	32	0.44	0.01	32	0.41	0.02
Ca _{Mean}	32	-0.75	< 0.001	32	-0.78	< 0.001	32	-0.77	< 0.001
MMR_NB-Tb.S	31	-0.46	0.009	31	-0.37	0.04	31	-0.35	0.05
CRYST_NB-Tb.S	31	0.54	0.002	31	0.51	0.003	31	0.50	0.003
MMR_NB-Tb.C	30	-0.67	< 0.001	30	-0.65	< 0.001	30	-0.63	< 0.001
CRYST_NB-Tb.C	30	0.43	0.02	30	0.47	0.008	30	0.48	0.007
HPP_NB-Tb.C	30	0.33	0.08	30	0.42	0.02	30	0.40	0.03

AdV/MaV: adipose volume to marrow volume ratio; BV/TV: bone volume to total volume ratio; Ca_{Width}: full-width at half maximum of the calcium distribution; Ca_{Peak}: most frequently occurring calcium concentration; F_{Peak}: bone surface frequency of the most frequently occurring calcium concentration; Ca_{Mean}: average calcium concentration; MMR: mineral-to-matrix ratio; CARB: relative carbonate B content; CRYST: mineral crystals' crystallinity; COLL: collagen maturity; HPP: hydroxyproline/proline ratio; GAG: glycosaminoglycans; NB-Tb.S: new bone – trabecular surface; NB-Tb.C: new bone – trabecular core; \$ indicates the correlation. N = sample size. The values are expressed as the Pearson correlation coefficient (r) and significance (p).

340 Discussion

341 The main objective was to evaluate the spatial correlation between BMAT and bone quality in settings of OVX.

342 Secondary objectives included describing the long-term effects of ovariectomy on bone.

343 **The trabecular bone in the OVX model exhibits characteristics of** 344 **postmenopausal osteoporosis in terms of bone loss and composition**

345 Liu *et al.* reported a 73% decrease in proximal tibia density in 15-month-old ovariectomized (OVX) rats [20].

346 Our study found a 70% lower BV/TV ratio in OVX rats compared to controls, aligning with existing literature.

347 BMAT significantly increased (+257%) in OVX rats. Limited animal model literature exists on BMAT, but in

348 humans, Li *et al.* observed greater BMAT in osteoporotic women (+7%, n=51) [21], with Sheu and Cauley

349 noting BMAT values from 8% to 45%. Our results indicated a more pronounced difference in rats after 9 months

350 post-ovariectomy. Coutel *et al.* introduced analysis of BMAT distribution, showing a significant increase in

351 adipocytes (AdV/MaV) near the bone surface in OVX rats ($p < 0.001$) [8], consistent with our results.

352 Regarding bone mineralization, Brennan *et al.* found a 5% lower mean calcium content (Ca_{Mean}) in the proximal

353 femur of OVX sheep [22]. We observed a 7% reduction in Ca_{Mean} in OVX rats, confirming the long-term effects

354 of ovariectomy. Valenta noted no significant Ca_{Mean} difference but a 14% reduction in full width at half

355 maximum (Ca_{width}) in OVX rats, suggesting long-term impacts on bone [23].

356 The MMR and the CARB reflect bone quality. Models have shown decreased MMR and increased CARB in

357 osteoporosis [24,25]. Our study confirmed lower MMR (-7%) and a non-significant 4% increase in CARB in

358 OVX rats, indicating reduced bone quality. Paschalis *et al.* found higher COLL and CRYST in osteoporotic

359 women, with lower GAG [26]. We observed higher CRYST (+1%) in OVX rats, but no significant changes in

360 COLL or GAG. HPP, linked to collagen modifications, was not significantly altered, though previous studies

361 reported increases [27]. We found a 7% increase in HPP in OVX rats. The observed increase in CRYST between

362 the SHAM and OVX groups, though relatively small, falls within the range of previous studies and may suggest

363 a subtle biological relationship, especially given the sensitivity of our measurements at the micrometer scale.

364 Among 22 OVX samples, only 7 contained old bone in the trabecular core (OB-Tb.C) and 3 contained old bone

365 on the trabecular surface (OB-Tb.S). Twelve OVX sample did not have OB-Tb.C and OB-Tb.S bone packets for

366 Raman analysis. This suggests less trabecular bone surface (see Fig. 5b) and mature bone in osteoporosis,

367 complicating analysis in small species like rodents. Our OVX model results align with existing literature on bone

368 quality and BMAT [28], validating its use in studying postmenopausal osteoporosis.

BMAT is correlated with the mineral component of trabecular bone quality

BMAT is negatively correlated with volumetric bone mineral density (vBMD) in postmenopausal osteoporotic women [29]. BMAT, a unique fat reservoir within bone cavities, is associated with obesity, which in turn is positively correlated with bone mineral density (BMD) [30]. However, fracture risk is higher in obese individuals, potentially due in part to BMAT's negative impact on bone quality and quantity [31]. Our study revealed a negative correlation between BMAT and bone microarchitecture.

Schwartz *et al.* (2013) also reported this negative correlation between BMAT and vBMD in postmenopausal women [29]. However, research on BMAT's interaction with bone quality at the trabecular level is limited. Our study aimed to fill this gap. We found that the AdV/MaV ratio correlated with bone mineral density distribution (BMDD) parameters. Increased BMAT was linked to decreases in Ca_{Peak} , Ca_{Mean} , and Ca_{Width} , and an increase in F_{Peak} , indicating a shift towards less mineralized and potentially weaker bone. This correlation was significant in new bone, suggesting a disrupted bone remodeling.

Due to limited correlations observed within the OVX group, our subsequent analysis and discussion focused on the entire sample. We carefully checked the data for consistency to ensure a cautious interpretation of our findings.

CRYST was positively associated with BMAT in new bone of pooled data over both groups (SHAM and OVX), suggesting adipocytes could influence apatite crystal maturation. This suggests that adipocytes and their secreted products may influence apatite crystal maturation through lipid metabolism's effect on mineralization, secretion of bone-modulating factors, and alteration of bone remodeling dynamics [32,33]. No significant correlation was found in old bone, likely due to substantial bone loss and increased remodeling rates, reducing old bone amount in OVX samples.

Our study showed a decrease in old bone surface ratio from 47% in the SHAM group to 13% in the OVX group. While we quantified this reduction, we previously also noted increased BMAT in an OVX context [8]. The degree of unsaturation of fatty acids in BMAT affects its lipotoxic impact on bone quality [33].

In this study, increased BMAT correlated with altered mineral content, decreased Ca_{Peak} and Ca_{Mean} in trabecular bone, and decreased MMR in new bone. Although the direct impact of adipocytes on bone quality is underexplored, recent evidence presents conflicting views. Entz *et al.* highlighted variations in BMAT's extracellular matrix, suggesting an influence on bone mineral quality [34], while other research offers counterarguments that moderate this perspective [35].

399

Limitations

400 The main objective was to evaluate the spatial relationship between BMAT and bone quality in settings of OVX.

401 Our results were in agreement with previous studies confirming the biological response of the animals to the

402 surgery. Despite the coherence of our results, the study has some limitations. As animal model, it has its own

403 obvious limits, as it cannot truly reproduce the human osteoporosis (e.g. no fragility fracture in the animal

404 model).

405 Considering the analytical techniques, outcomes from Raman, qBEI, and μ CT are surrogates to evaluate the

406 mineral content and bone marrow, and should be taken with caution. The relative bone tissue age was

407 approximated by qBEI which is not the gold standard method and may induce some variability in the Raman

408 outcomes. The use of fluorescent label would improve the evaluation of tissue age. However, our Raman results

409 were found in agreement with previous studies. It supports that our approximation of tissue age was done

410 correctly.

411 Our results concerning bone marrow are mainly representative of the regulated bone marrow adipose tissue

412 (rBMAT) and not constitutive BMAT due to the selection of the proximal tibial red marrow as an analysis

413 region.

414 The lack of significant correlations in our study likely reflects limitations such as small sample size, data

415 variability, and uncontrolled confounding factors, rather than an absence of a true relationship between BMAT

416 and bone quality. In complex systems like the OVX/osteoporosis model, correlations may not always be evident,

417 underscoring the need for further research with larger, more controlled studies.

418 **Conclusion**

419 In conclusion, we investigated bone quality at various scales, including mineral bone density distribution and
420 bone ultrastructural composition, in relation to BMAT changes in the proximal metaphysis of tibiae in setting
421 OVX. Our findings were in agreement with previous studies, such as reduced microarchitecture and mineral
422 content, structural alterations in the mineral component, and increased bone marrow adipose tissue. Additionally,
423 we examined the correlations between BMAT and bone quality in setting OVX, considering the spatial
424 distribution of adipocytes. Interestingly, BMAT was found to be associated with parameters of the mineral
425 component, including bone mineral density distribution, the mineral-to-matrix ratio, and hydroxyapatite crystal
426 perfection. As BMAT increased, these parameters shifted toward an impaired bone quality, especially near the
427 trabecular newly formed bone areas, as identified by the qBEI. These findings provide new insights for further
428 investigations into the relationship between BMAT and bone tissue during postmenopausal osteoporosis.

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