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Mandibular bone is protected against microarchitectural alterations and bone marrow adipose conversion in ovariectomized rats

Xavier COUDEL*¹, Jérôme DELATTRE¹, Pierre MARCHANDISE¹, Guillaume FALGAYRAC¹, Hélène BEHAL², Greet KERCKHOFS^{3,4,5,6}, Guillaume PENEL¹, Cécile OLEJNIK¹,

1. Univ.Lille, Univ. Littoral Côte d'Opale, CHU Lille, EA 4490 - PMOI, F-59000 Lille, France
 2. Univ. Lille, CHU Lille, EA 2694 - Santé publique: épidémiologie et qualité des soins, Unité de Méthodologie et Biostatistiques, F-59000 Lille, France
 3. Biomechanics lab, Institute of Mechanics, Materials, and Civil Engineering, UCLouvain, Louvain-la-Neuve, Belgium
 4. Institute of Experimental and Clinical Research, UCLouvain, Woluwe, Belgium
 5. Department Materials Engineering, KU Leuven, Leuven, Belgium
 6. Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, Belgium
- Corresponding author e-mail: xavier.coutel@univ-lille.fr

ABSTRACT

Osteoporosis is a disease that leads to a loss of bone mass and to alterations in the bone microarchitecture that occur in a site-specific manner; however it remains controversial in the jaw. The involvement of bone marrow adipose tissue (BMAT) in the bone metabolism has been suggested in several physiopathological contexts, such as in aging and osteoporosis. To test whether the BMAT content is related to mandibular bone loss, this study aimed to investigate the potential correlations between the trabecular bone microarchitecture on one hand and BMAT content and its spatial distribution in relation to bone surface on the other hand during aging and ovariectomy (OVX) during a long-term follow-up in a mature rat model.

No age-related microarchitectural or BMAT changes were observed in the mandible. The OVX-induced bone loss was three-fold lower in the mandible than in the tibia and was observed only in the alveolar bone (not in the condyle). We also report a delayed increase in the mandibular BMAT content that remained 4-6-fold lower compared to tibia. This low BMAT content in the mandible was located at a distance from the trabecular bone surface (only 5% in contact with the bone surface versus 87% in the tibia). These findings highlight a specific mandibular response to OVX, in particular fewer microarchitectural alterations compared to that in the tibia. For the latter, the trabecular bone thickness and surface were correlated with the BMAT content. Oral functions may have a protective effect on the mandibular BMAT conversion in an OVX context.

Keywords: Rats; Mandible; Ovariectomy; Aging; Bone microarchitecture; Bone Marrow
Adipose Tissue; Micro-CT

1 INTRODUCTION

2 Osteoporosis is a common and widespread skeletal disease that is characterized by an
3 imbalance of bone remodeling that leads to a systemic loss of bone mass and in changes to
4 the bone microarchitecture that occur in a site-specific manner ¹. The loss of gonadal
5 function and aging are the two most important factors contributing to the development of
6 the structural decay of the trabecular and cortical bone ². These skeletal alterations, which
7 are both quantitative and qualitative, are most often described at the skeletal sites of
8 fractures, such as the long bones of the appendicular skeleton (the epiphyses of the femur
9 and the tibia in particular) and at the bones of the axial skeleton (vertebrae) ³. Although
10 several clinical studies have reported correlations between the changes in the bone mineral
11 density (BMD) at the hip or spine, and in the changes in the alveolar and basal BMD in the
12 mandible, other studies have shown conflicting results in an osteoporosis context ^{4,5}. In an
13 ovariectomized rat model (mostly in young animals), a lower response to estrogen deficiency
14 has been reported in the mandibular alveolar bone compared to that in the proximal
15 metaphysis of the tibia, especially in the trabecular bone compartment ^{6,7}. Hence, the
16 existence of osteoporosis of the cranio-facial skeleton and its impact on the teeth-bearing
17 areas (alveolar bone) are still debated today ^{8,9}. In particular, there is still a lack of
18 information on the bony changes in aged animals (mature skeleton) ¹⁰ and their
19 relationships with local factors, such as bone marrow adipose tissue (BMAT) content and
20 spatial distribution, whose potential regulatory roles in bone metabolism have been paid
21 progressively more attention in the field ¹¹.

22

23 We previously showed that the mandibular trabecular bone had a high density compared to
24 that of the tibia which, in contrast, has a large surface area that is in contact with the bone
25 marrow ¹². In addition to their close anatomical vicinity, the cells of the bone and the BMAT
26 are critical for each other's functions that ensure bone homeostasis ¹³. Bone marrow
27 adipocytes (BMAds) are the most abundant differentiated stromal cells in human adult bone
28 marrow, representing up to 70% of the marrow volume or 5-10% of the total weight of the
29 whole body fat mass ¹⁴. The hematopoietic cellularity of bone marrow decreases with age
30 with a corresponding increase in the fat fraction ¹⁵. Bone marrow adipose conversion,
31 resulting from the differentiation of skeletal stem cells (SSCs), appears to be a tightly

32 regulated process ¹⁶. The BMAT content has been reported to vary in several
33 physiopathological contexts, such as aging ¹⁷ or postmenopausal osteoporosis ^{18,19}, and it has
34 also been reported to be inversely correlated to the trabecular bone volume and resorption
35 surface in iliac crest bone biopsies, thus playing a key role in the regulation of bone turnover
36 ^{20,21}. Several magnetic resonance imaging (MRI) studies have reported, in osteoporotic
37 women, a higher bone marrow fat fraction (BMFF), mainly in the vertebrae and the long
38 bones ^{17,22}. This increase was found to be inversely related to bone formation (osteoblastic
39 activity) during osteoporosis, supporting the hypothesis that a preferential differentiation of
40 SSCs toward the adipogenic pathway occurs at the expense of the osteogenic pathway ¹⁸. In
41 addition, a decrease in the unsaturation index of the BMFF has also been reported ¹⁹, as well
42 as an increase in the content of pro-inflammatory cytokines (i.e., IL-6 and TNF α) and
43 adipogenic markers (i.e., adiponectin and soluble RANKL) within the bone marrow
44 supernatant fluid of osteoporotic patients, leading to increased bone fragility ²³. The bone
45 marrow adiposity (BMA) is thus recognized as a major metabolic actor in the
46 pathophysiology of osteoporosis ^{24,25}.

47

48 The distribution of BMAT in the skeleton reveals site specificities ²⁶⁻³⁰. In rodents, the
49 content and composition of BMAT have been reported to be distributed differently between
50 the distal and proximal metaphysis of the tibia ³¹ and also between the lumbar and caudal
51 vertebrae ³². In addition, a preferential location of BMAdS has been reported among human
52 iliac crest bone biopsies in different physiopathological contexts: stacked in the
53 paratrabecular areas in osteoporosis and in the central bone marrow areas in plasmocytoma
54 ³³. Moreover, bone marrow stromal cell (BMSC) progenitors residing close to the bone
55 surface in long bones (tibiae) have been reported to be functionally distinct from those in
56 the central bone marrow, suggesting a differential metabolic activity according to the
57 distribution pattern of the BMAdS ³⁴. In preclinical osteoporosis models, ovariectomy-
58 induced trabecular bone loss was observed with distinct patterns according to the location
59 within the tibia ²⁹. In addition, BMAT increased with osteoporosis, while the trabecular bone
60 volume decreased, suggesting an inverse relationship between osteogenesis and
61 adipogenesis ^{16,18} and arguing for an involvement of BMAT in the impairment in the
62 osteoresorption-osteof ormation balance ^{35,36}.

63

64 Mandibular bone loss remains understudied and could be triggered, during aging and
65 osteoporosis, by variations in the BMAT content and composition ³⁷. BMAT could represent
66 an early biomarker of the occurrence and progression of the osteoporotic status, and it has
67 been insufficiently explored clinically ^{22,37,38}. In the present study, we used an original
68 methodology for the simultaneous 3D assessment of the bone microarchitecture ³⁹ BMAT
69 content and spatial distribution ¹². The objectives of the present study were as follows: i) to
70 assess the long-term changes in the mandibular bone microarchitecture, BMAT content and
71 spatial distribution during aging and after ovariectomy in a mature rat model and to
72 compare these parameters with the proximal metaphysis of the tibia, and ii) to analyze
73 whether these parameters are correlated within the functional alveolar and condylar areas
74 of the mandible. In addition, differences in the masticatory activity between the
75 experimental and control groups were taken into account by standardizing the food intake.
76 This standardization was achieved by correcting of the transient hyperphagia in the
77 ovariectomized animals by setting a long-term pair-feeding protocol consisting of providing
78 equal food intake with that of the SHAM groups. Thus, the specificities between the alveolar
79 bone and condylar regions within the mandible were explored in conditions where the diet
80 was controlled (OVX _PF groups) or not (OVX_AL groups).

81 MATERIAL AND METHODS

82 Animal Procedures

83 Six-month-old female Sprague-Dawley rats (*rattus norvegicus*, n=69) were used in this study
84 (Janvier Lab, Laval, France). The provider reported that the rats were free of known viral,
85 bacterial and parasitic pathogens. Animals were randomly assigned prior to the operation by
86 the provider in three distinct groups: the OVX_AL (n=20), OVX_PF (n=20) and SHAM groups
87 (n=29). OVX_AL and SHAM groups were fed *ad libitum* (free access to tap water and a
88 standard diet). A pair-feeding protocol was maintained until the sacrifice of the animals. All
89 experiments on animals were carried out with the approval of the French Ministry of
90 Research and Innovation (approval no. APAFIS#4197-2015091514435707). Animal
91 procedures were performed in an accredited animal facility (authorization no. B5935010) at
92 the University of Lille with the approval of the Animal Ethics Committee and in accordance
93 with the directive 2010/63/EU and the “Principles of Laboratory Animal Care” recommended
94 by the National Society for Biomedical Research in France. Rats (n=3/cage according to the
95 EU laws) were housed in type 4 cages filled with Lignocel® (hygiene animal bedding) enriched
96 with horizontal tubes for climbing and were kept under controlled conditions at 22±2°C on a
97 12 h light/12 h dark cycle with lights on from 07.00 a.m to 7.00 p.m. Rats were euthanized by
98 exsanguination under anesthesia at 6 months (baseline, n=9), 10 months (M4 post-OVX, n=9)
99 and 15 months of age (M9 post-OVX, n=11). At sacrifice, the right hemimandibles and tibiae
100 were harvested, immediately fixed for 48 h in 10% neutral-buffered formalin and stored in
101 phosphate buffered saline prior to further micro-CT analyses.

102

103 Micro-CT image acquisition and reconstruction: SCAN1

104 To simultaneously assess the bone microarchitecture and the BMAT content, the trabecular
105 area was investigated in three skeletal locations: (i) the intraradicular alveolar bone of the
106 first molar; (ii) the central area of the mandibular condyle in the mandibles and (iii) the
107 secondary spongiosa of the tibial metaphysis. All bone samples were scanned for the first
108 time (i.e., SCAN1) to allow the analysis of the trabecular bone morphometric parameters.
109 Specimens (mandibles and tibiae) were placed in a plastic sample holder and were scanned
110 using a Skyscan 1172 micro-CT device (BRUKER, Kontich, Belgium) with the software suite

111 provided by the manufacturer. Scan settings were as follows: isotropic voxel size $10\mu\text{m}^3$, 80
112 kVp, 100 μA , Al-Cu filter, 2400 ms integration time, and 0.5° rotation step over 180° . For the
113 reconstruction, the following settings were applied: Gaussian smoothing, ring artifact
114 reduction 6, and beam hardening correction 50%. After the first acquisition, the samples
115 were prepared for BMAT analysis ¹² and were scanned a second time (SCAN2) to allow the
116 analysis of the BMAT parameters using the same acquisition settings as the first scan. Briefly,
117 each sample was decalcified and stained in a 1% osmium tetroxide solution (stabilized with a
118 2.5% potassium dichromate solution).

119

120 **Osmium tetroxide staining protocol**

121 After SCAN1, the samples were prepared for BMAT analysis. Thus, all the bone samples were
122 decalcified under controlled slow oscillations in 4% formic acid/10% NBF (1:1), pH 7.4, for 4
123 days. Once the bones were demineralized, they were rinsed in distilled water. Each
124 decalcified bone was then stained in the fume hood for 48 h in an aqueous staining solution
125 that was composed of a 1% osmium tetroxide solution that was stabilized in a 2.5%
126 dichromate potassium solution at room temperature. Then, the stained bones were rinsed
127 twice in PBS for 3 h each at room temperature and a last wash was performed in the hood
128 overnight. Osmium stained bones were then moved to a fresh set of 50 ml falcon tubes
129 containing 45 ml of PBS each and were stored at 4°C until the micro-CT acquisition.

130 **Micro-CT image acquisition and reconstruction: SCAN 2**

131 After osmium tetroxide staining, the decalcified and stained bone samples were scanned a
132 second time (i.e., SCAN2) to allow the analysis of the BMAT. Samples were scanned using the
133 same acquisition settings as the first scan. For the reconstruction of the SCAN2 datasets, the
134 following settings were applied to reveal the osmium-stained BMAT: Gaussian smoothing,
135 ring artifact reduction 3, and beam hardening correction 30%.

136 **Image datasets alignment (SCAN1 and SCAN2 registration)**

137 The SCAN1 and SCAN2 datasets were rotated to obtain the same orientation, which was
138 specific for each skeletal location (the longitudinal axis of the proximal metaphysis in the
139 tibia and the mandibular condyle and the first molar in the alveolar ridge for the mandible).

140 Then, the reoriented datasets of each sample (SCAN1 as the reference, SCAN2 as the target)
141 were manually registered using the Dataviewer software (v. 1.5.2.4) following these critical
142 steps: (i) the coregistration of the global outlines; (ii) the adjustment of the enamel of the
143 rodent incisor (in the case of the alveolar ridge) and (iii) the progressive adjustment of the
144 trabecular network and bone marrow space using the profile scale diagram and different
145 angles of view.

146

147 **Determination of the Regions of Interest (ROI)**

148 After registration, the regions of interest (ROI) were drawn in the SCAN1 datasets that
149 delineated the trabecular area of the samples using CTAn. For the alveolar ridge, the ROIs
150 were selected as the intraradicular bone of the first molar from the furcation to the apex
151 roots, as adapted from the protocol of Du et al. (modification: an oval shape was used
152 instead of a circular one) ⁴⁰. In the condyle, the ROI was selected according to the method
153 described by Jiao et al., as a rectangle shape (1 mm length, and 0,5 mm height) in the central
154 compartment ⁴¹. In the proximal tibia, 200 cross-sections (2 mm height) were selected 1.5
155 mm under the growth plate, and the trabecular area was isolated from the cortical area by a
156 semiautomated protocol in CTAn[®] ⁴².

157

158 **Micro-CT image analysis - trabecular bone microarchitecture**

159 In the ROIs of the SCAN1 datasets, a one-level Otsu's method was used to determine the
160 threshold between the mineralized tissue and the bone marrow compartment (and vice
161 versa) ⁴³, and the following bone microarchitectural parameters, reflecting the morphology
162 of the trabecular network for each skeletal location, were measured using CTAn[®]: percent
163 bone volume (BV/TV, %), trabecular bone surface/volume ratio (BS/TV, mm⁻¹), trabecular
164 number (Tb.N, mm⁻¹), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm)
165 and trabecular pattern factor (Tb.Pf, mm⁻¹).

166

167 **Micro-CT image analysis – BMAT content and distribution**

168 For the quantification of the BMAT content, each defined ROI of the SCAN1 datasets was
169 reused on the coregistered SCAN2 dataset to determine the BMAT content in the same area
170 that the bone microarchitecture was assessed. For the mandibular bone, a fixed-threshold
171 value (between 110 and 255 grayscale levels) was used for both skeletal locations. In the
172 tibia, the BMAT was segmented using a multilevel Otsu algorithm (3 levels). The following
173 BMAT parameters were measured for each skeletal location: the percentage of adipocyte
174 volume in the total volume of the ROI (Ad.V/TV, %) and, in using an original method, the
175 ratio adipocyte volume/marrow volume (Ad.V/Ma.V, %). To assess the spatial distribution
176 profile of BMAT in relation to the bone surface, a customized stepwise process was created
177 in CTAn[®] to plot the percentage of BMAT vs the distance from the trabecular bone surface.
178 After uploading the segmented osmium-stained BMAT from the SCAN2 datasets as ‘the
179 image’ and the binarized-trabecular network of the SCAN1 datasets as ‘ROI’, this task list
180 combines the repetition of the following tasks 5 times: a one-level Otsu thresholding, a
181 morphological operation (20 μ m 3D dilatation step = 2 pixels), a saving image and a 3D
182 morphological analysis to determine the percentage of BMAT in each volume.

183 **Statistical analyses**

184 All statistical analyses were performed with the SAS package software[®], release 9.4 (SAS
185 Institute, Cary, NC). Continuous variables are described by the mean and standard deviation,
186 or the median and interquartile range. The normality of the distributions was evaluated
187 graphically and using the Shapiro-Wilk test. Animal body weight was compared between the
188 groups, week by week, using a linear mixed model to account for the correlation between
189 the repeated measures within subjects by including a random subject effect.

190 The parameters of the trabecular bone microarchitecture and BMAT content were compared
191 among the three time points (baseline, M4, M9) in the SHAM group for each skeletal
192 location (the proximal metaphysis of the tibia, alveolar bone and condyle) with a linear
193 mixed model with a random subject effect.

194 The parameters of the bone microarchitecture and BMAT were compared among the three
195 groups (SHAM, OVX_AL, OVX_PF) within each skeletal location and at M4 and M9 with a
196 linear mixed model with a random subject effect. In the case of significant results, post hoc
197 comparisons between the groups were performed.

198 Associations between the trabecular bone microarchitectural parameters and BMAT
199 parameters were assessed for each skeletal location using the Spearman correlation
200 coefficient in the SHAM group and in the pooled OVX groups.

201 The spatial distribution of the BMAT content was compared among the groups by
202 localization and time (M4 and M9) using an analysis of covariance model adjusted for the
203 animal's weight. In the case of significant results, post hoc comparisons between the groups
204 were performed. The statistical significance for all tests was set at $p < 0.05$.

205

206 **Principal Component Analysis**

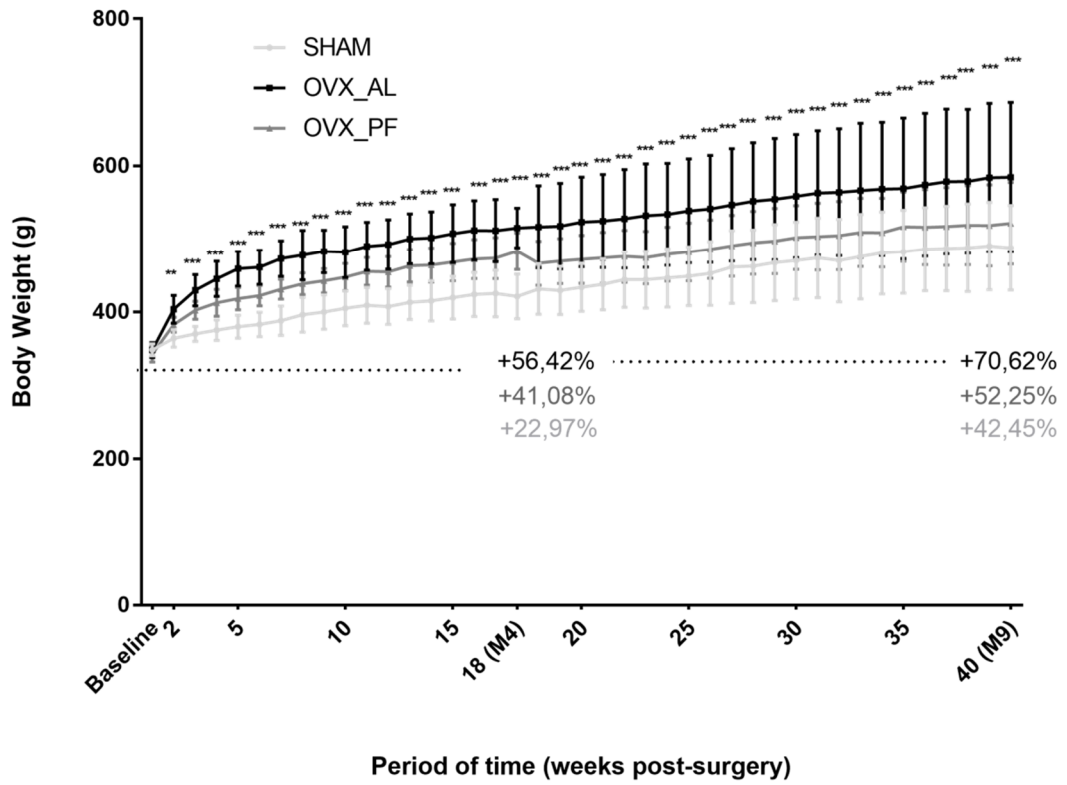
207 The micro-CT data were processed with Principal Component Analysis (PCA) ⁴⁴. PCA is
208 described as a "unsupervised" method that explores the variability within a dataset. This
209 statistical multivariate analysis tool allows for the study of the relationships among the
210 variables (BV/TV, Ad.V/Ma.V etc.) and the samples of the groups (SHAM and OVX) at the
211 different postsurgery observational times (baseline, M4, and M9). PCA was performed with
212 the PLS Toolbox v. 6.7 (Eigenvector Research Inc., West Eaglerock Drive, Wenatchee, WA,
213 98801) operating in the MATLAB R2010a environment (Mathworks Inc., Natick, MA, USA).
214 Prior to PCA, the autoscale function was applied to the dataset.

215

216 **RESULTS**

217 **Animal body weight**

218 The average weight of the animals before surgery was 346.3 ± 9 g (Fig. 1). From the second
219 week after OVX, a weight difference of approximately 10% was observed between the
220 animals of the OVX_AL group and those of the SHAM group (403.6 ± 19 g vs 363.9 ± 12 g, p
221 < 0.001), highlighting a rapid weight gain after OVX. This was followed by a slower weight
222 gain from week 8 to week 40 (after transient hyperphagia, which was observed until 8 weeks
223 post-surgery). This weight difference was not found between the OVX_PF and the SHAM
224 group at 2 weeks post-OVX (382.4 ± 10 g vs 363.9 ± 12 g, $p=0.10$).



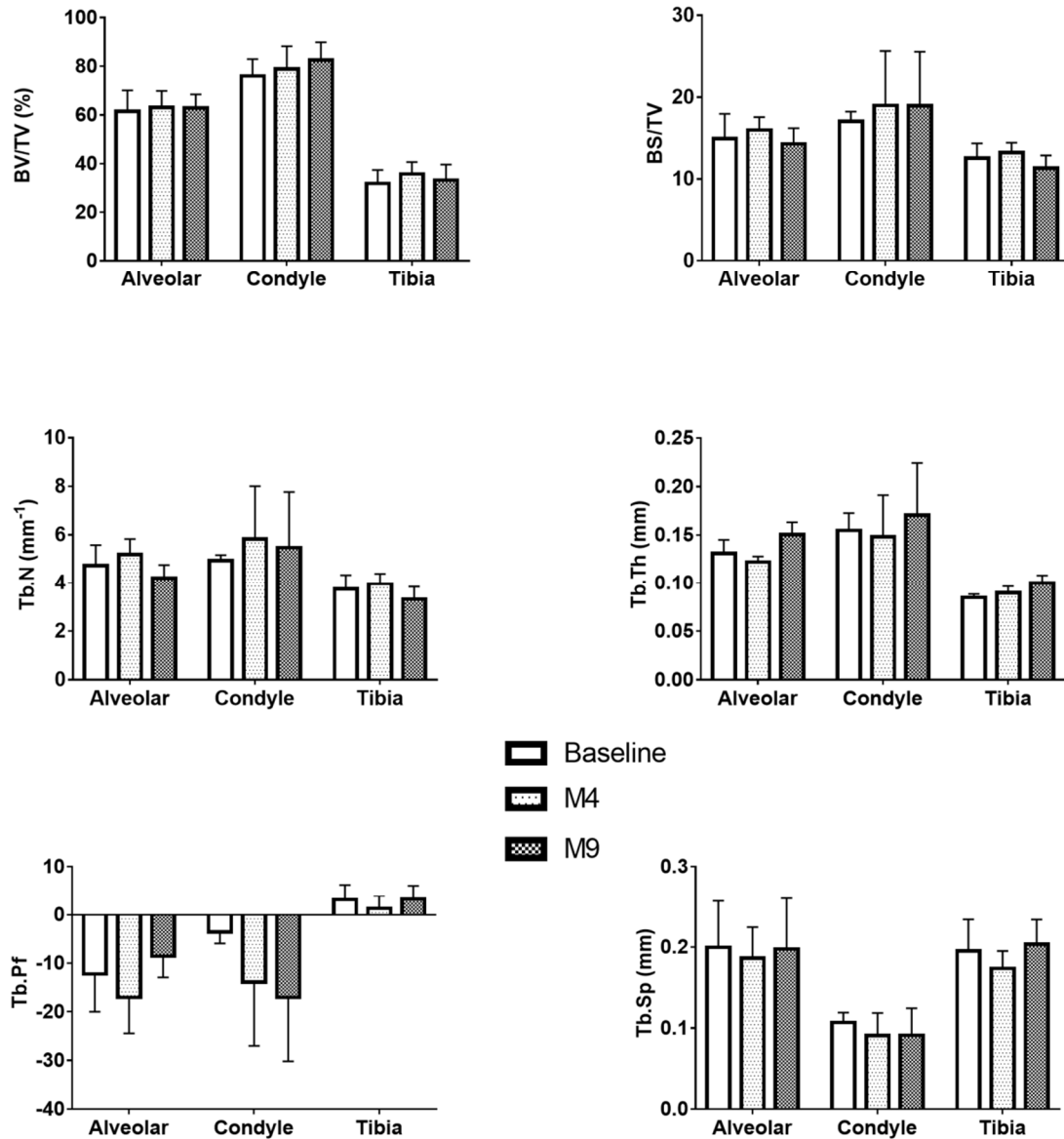
225

226 **Figure 1 : Animal body weight differences from week 0 to week 40 in SHAM, OVX_AL and**
 227 **OVX_PF rats. Values are means \pm SD. Differences between groups are shown: ** $p < 0.01$,**
 228 ***** $p < 0.0001$.**

229

230 **Trabecular bone microarchitecture**

231 In the SHAM group, no significant differences related to aging were observed in the
 232 trabecular bone microarchitectural parameters regardless of the skeletal location (alveolar
 233 bone, condyle, and tibia – Fig. 2).



234

235 **Figure 2 : No age-related effects on trabecular bone microarchitecture whatever skeletal**
 236 **sites (alveolar bone, condyle, tibia) in the SHAM groups respectively at baseline, M4 and**
 237 **M9. Values are means ± SD.**

238

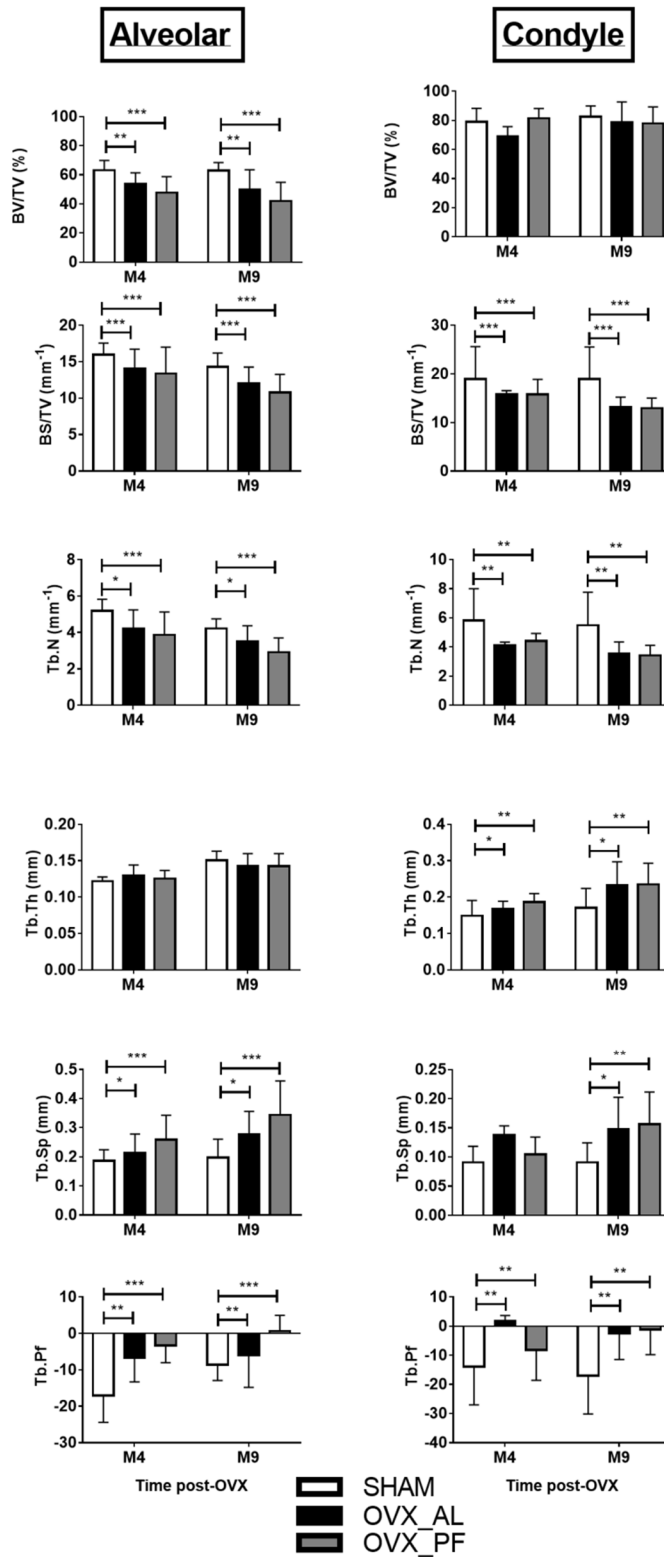
239 The trabecular bone microarchitectural parameters were significantly modified between the
240 SHAM and OVX groups according to the skeletal locations. However, no differences were
241 observed in the trabecular bone microarchitectural parameters between the OVX_AL and
242 OVX_PF groups at M4 and M9, regardless of the skeletal location (Figs. 3-Additional Fig. 8).
243 In the tibia, at M4, significant modifications in both of the OVX groups were observed
244 compared to those of their SHAM counterparts: the trabecular BV/TV was 62% lower, Tb.N
245 was 64% lower and both Tb.Th. (+10%, $p < 0.0001$) and Tb.Sp. were higher (+65%, $p < 0.001$) in
246 the OVX groups compared to those of the SHAM group. In addition, the Tb.Pf. was
247 approximately 10-fold higher ($p < 0.0001$) in the OVX group compared to that of the SHAM
248 group. No changes in the microarchitectural parameters were observed between M4 and
249 M9 (see Additional Fig. 8).

250

251 In the alveolar bone (Fig. 3), significant modifications in both of the OVX groups were
252 observed compared to those of their SHAM counterparts: BV/TV was 15% lower at M4 and
253 20% lower at M9 ($p < 0.0001$), Tb.N. was 19% lower at M4 post-OVX ($p < 0.0001$) in the OVX
254 groups compared to that of the SHAM group. The Tb.Th. did not change regardless of the
255 time post-OVX. The OVX groups were also characterized by a higher Tb.Sp. (+10% at M4,
256 $p < 0.0001$ and +28.5% at M9, $p < 0.0001$) compared to that of the SHAM group.

257

258 In the condyle (Fig. 3), no significant changes in the trabecular BV/TV were observed at M4
259 or M9 post-OVX. However, the OVX groups were characterized by a lower Tb.N. (up to -35%
260 at M9, $p < 0.001$) compared to that of the SHAM group. In addition, when comparing the OVX
261 groups to the SHAM group, we observed a higher Tb.Th. (+26% at M9, $p = 0.027$), which was
262 associated with a more than 6-fold increase in the Tb.Pf. ($p = 0.002$) and a higher Tb.Sp. at
263 M9 (+40%, $p = 0.040$).



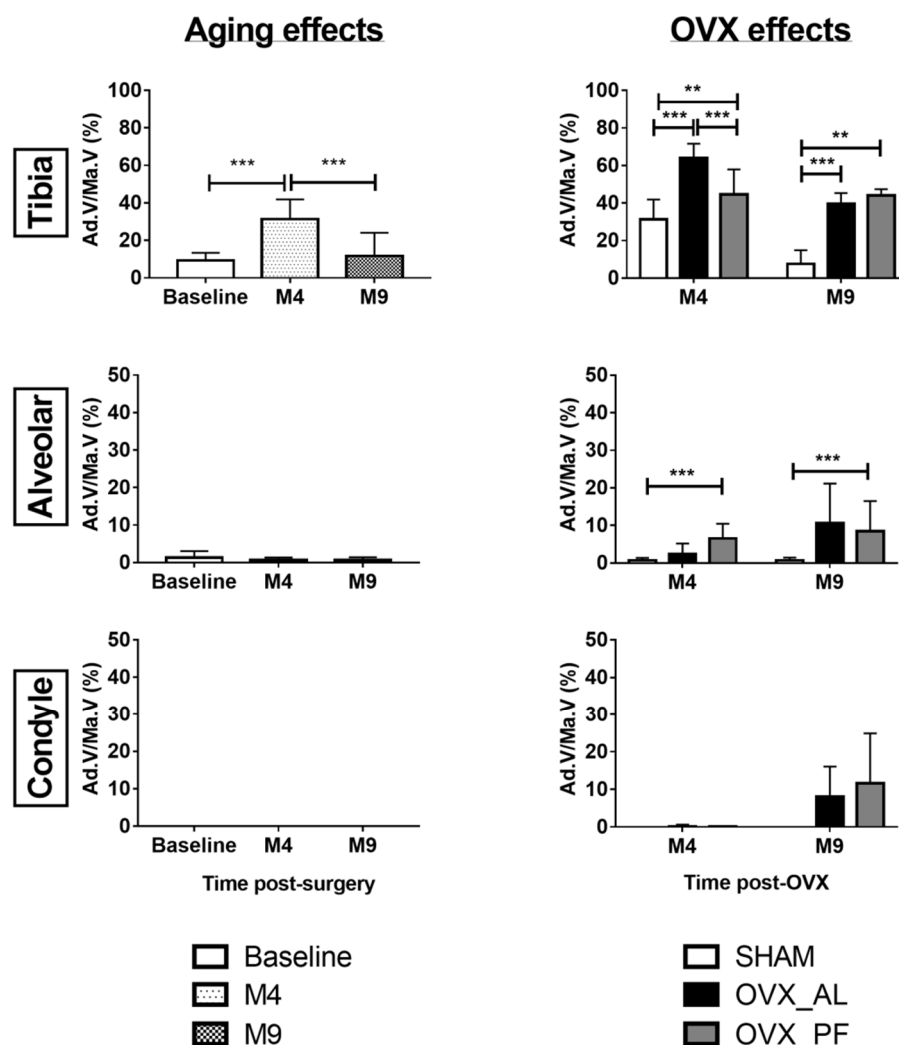
264

265 **Figure 3 : OVX-related effects on trabecular bone microarchitecture in the alveolar bone**
 266 **(left) and in the condyle (right) respectively at M4 and M9. Values are means ± SD.**
 267 **Differences between groups are shown: *p<0.05, **p<0.01, *** p<0.0001**

268

269 **BMAT content**

270 In the SHAM group (Fig. 4), no age-related changes in the BMAT content (Ad.V/Ma.V, %)
 271 were observed in both mandibular sites, but there was a 2-fold significant increase in the
 272 BMAT content in the tibia at M4 post-OVX compared to that at baseline and a significant
 273 decrease of -30% at M9 compared to that at M4 post-OVX.
 274 In the mandible, no BMAT content was found in the condyle regardless of the age of the
 275 animals in the SHAM group. Interestingly, at baseline, the BMAT content in the tibia
 276 exhibited a significantly 10-fold increase compared to that of the alveolar bone.



277

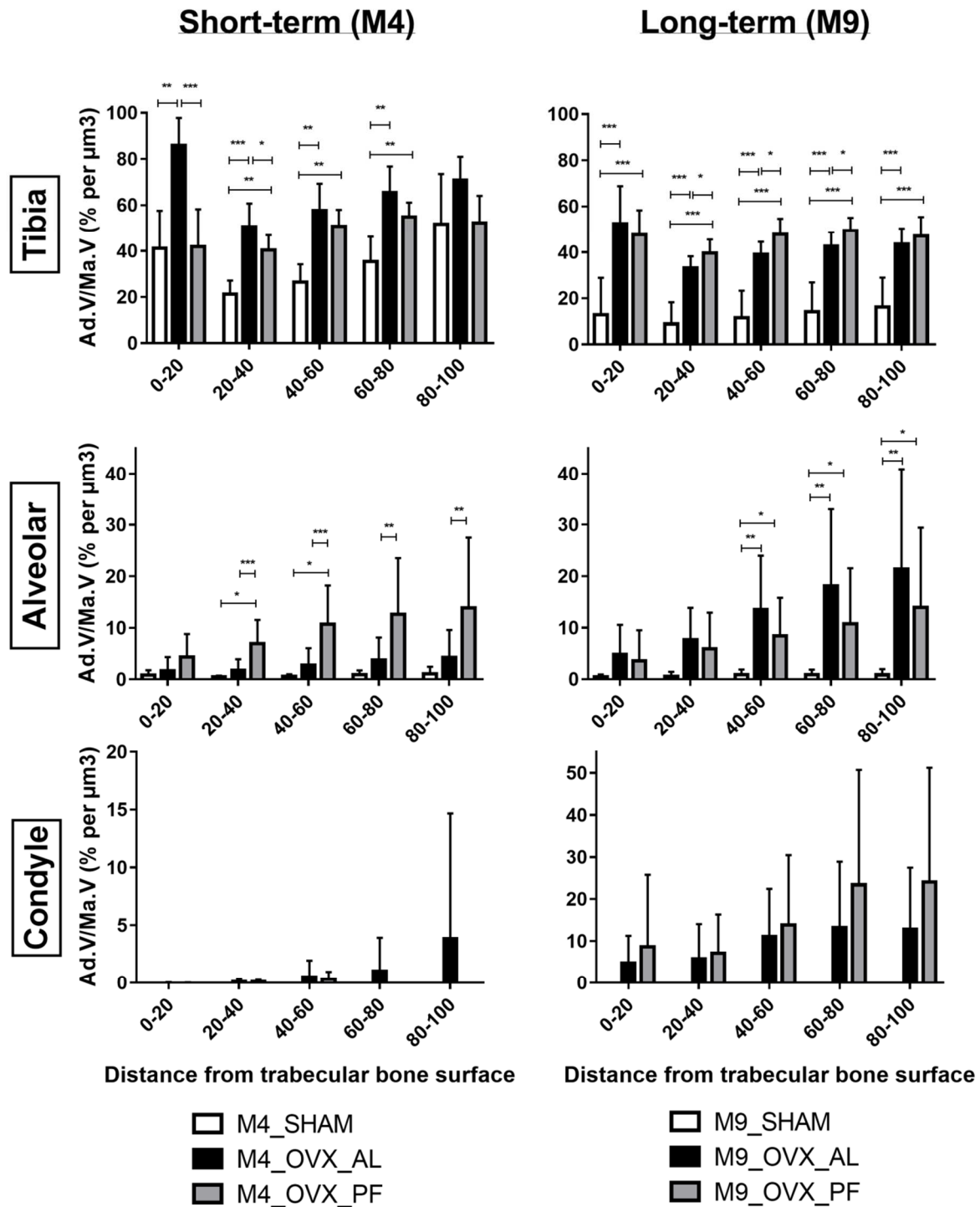
278 **Figure 4 : Age and OVX-related effects on BMAT content (Ad.V/Ma.V: adipose**
 279 **volume/marrow volume, %) respectively at baseline, M4 and M9 in the 3 skeletal locations**
 280 **(tibia, alveolar bone, condyle). Values are means ± SD. Differences between groups are**
 281 **shown: * p <0.05, ** p <0.01, *** p <0.0001**

282 After OVX (Fig. 4), the bone loss observed in the tibia was accompanied by an increase in the
283 BMAT content at M4 and M9 compared to that of the SHAM group. Indeed, there was a
284 significant increase in the Ad.V/Ma.V compared to that of the age-matched SHAM group
285 (+51% at M4, $p < 0.0001$ and +337% at M9, $p < 0.0001$). In the alveolar bone, bone loss was
286 accompanied by a significant increase in the Ad.V/Ma.V compared to that of the age-
287 matched SHAM group, but this effect was only observed in the OVX_PF group (+1908% at
288 M4, $p < 0.0001$, and +1256% at M9, $p < 0.0001$). In the condyle, no bone loss was found, but
289 small amounts of adipose content appeared at M4 post-OVX (approximately 1% of the
290 available marrow volume) and reached 10% at M9 post-OVX. The micro-CT 3D renderings of
291 the alignment of the bone microarchitecture and BMAT content are illustrated at each
292 skeletal site during aging and OVX in additional figures 9-10-11.

293

294 **BMAT spatial distribution**

295 Figure 5 illustrates the spatial distribution of BMAT within the proximal tibia, alveolar bone
296 and condyle, which was calculated as the distance from the trabecular bone surface, up to
297 100 μm away¹². In the tibia, BMAT was preferentially in contact with the trabecular bone
298 surface after ovariectomy. Indeed, 87% of the trabecular bone surface was covered by
299 BMAds at M4 in OVX_AL group versus 40% in the SHAM group ($p = 0.008$). A higher BMAT
300 content was found in contact with the trabecular bone surface at M9 in both OVX_PF group
301 (48%) and OVX_AL group (52%) compared to the SHAM group (13%, $p < 0.0001$). In contrast,
302 in both mandibular sites, the small amount of BMAT remained distributed within the entire
303 marrow spaces with less than 5% of the trabecular bone surface covered by BMAds
304 whatever the time post-OVX.

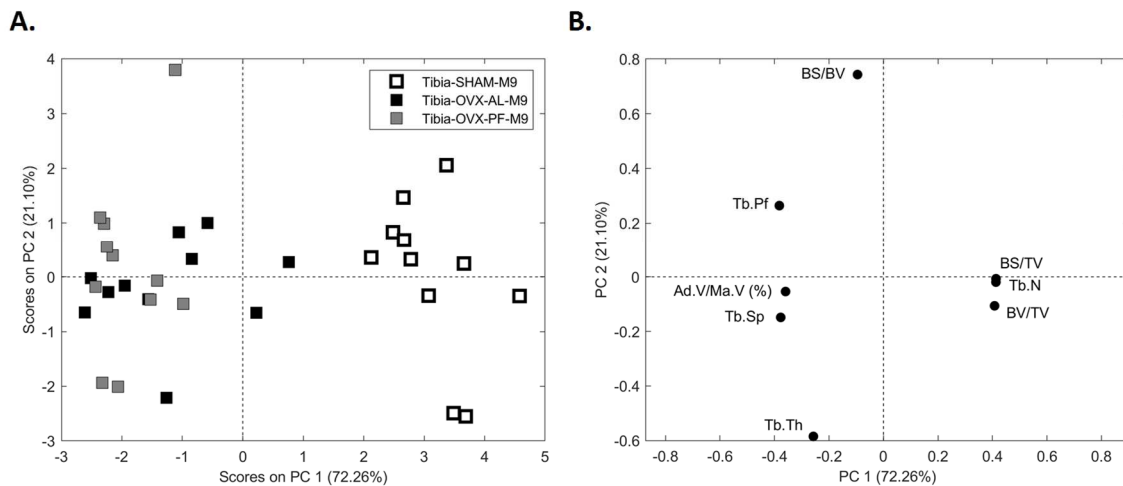


305

306 **Figure 5 : BMAT spatial distribution at M4 (left) and M9 (right) for each skeletal locations.**
 307 **Values are means \pm SD. Differences between groups are shown: * $p < 0.05$, ** $p < 0.001$,**
 308 ***** $p < 0.0001$**

309 **Relationship between bone microarchitecture and BMAT variables**

310 The global relationship among the micro-CT variables and groups (SHAM and OVX) was
311 explored with principal component analysis (PCA). In the tibia, the score plot (Fig. 6A) shows
312 two clusters that were formed by the SHAM group (right) and the OVX groups (left). This
313 separation was made along PC1 (72.26%). No PCA-based separations between the OVX
314 groups were observed. The loading plots (Fig. 6B) show that the BV/TV, BS/TV and Tb.N were
315 higher in the SHAM group than in the OVX groups. The values of the Tb.Pf, Tb.Sp, Tb.Th, and
316 Ad.V/Ma.V were higher in the OVX groups than in the SHAM group.
317 The PCA was also conducted on the datasets related to the mandibular variables. However,
318 no clear and distinct separation was observed at either mandibular site.



319

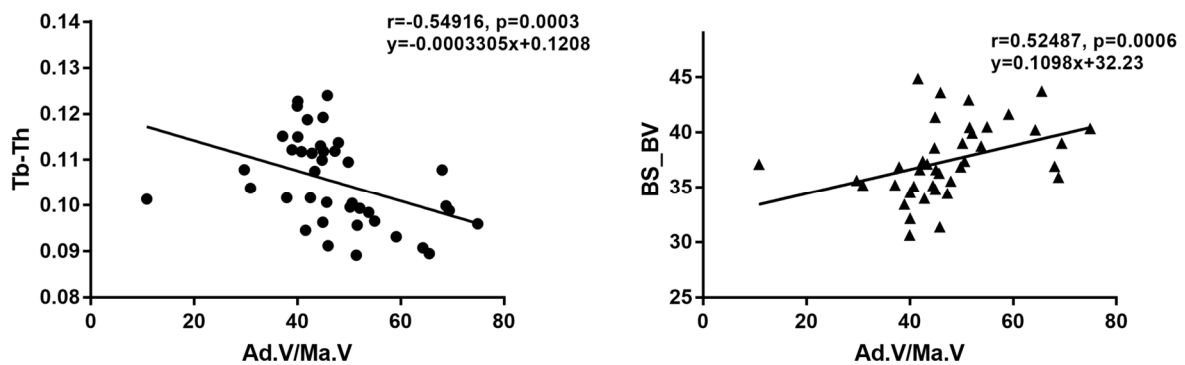
320 **Figure 6 : Principal Component Analysis (PCA) in the tibia (M9).**

321 **A. Score plots, representing the distribution of samples in the space defined by PC1 and**
322 **PC2, shows 2 distinct clusters associated to SHAM group (right) and OVX groups (left)**

323 **B. Loading plots, representing the contribution of each variable in the space defined by**
324 **PC1 and PC2, shows the association of micro-CT variables that most characterized the**
325 **SHAM groups (right) and both OVX groups (left).**

326

327 Linear regression analysis (bivariate approach) was then carried out to explore the
328 correlations between the microarchitectural and BMAT variables in the OVX and SHAM
329 groups, at the various time-points postsurgery. No correlations among the bone
330 microarchitectural and BMAT parameters were found in the SHAM group (data not shown).
331 However, in the OVX groups, for the tibia only, significant correlations were found between
332 the Ad.V/Ma.V and Tb.Th ($r=-0.55$, $p=0.0003$) and between the Ad.V/Ma.V and BS/BV
333 ($r=0.52$, $p=0.0006$) (Fig. 7).



334

335 **Figure 7 : Negative correlation between Tb.Th and Ad.V/Ma.V and positive correlation**
336 **between BS/BV and Ad.V/Ma.V in the tibia among both OVX groups (OVX_AL and**
337 **OVX_PF). The correlation coefficient, level of significance p and equation of the line are**
338 **indicated (nonparametric Spearman rank correlation test).**

339 **DISCUSSION**

340 The ovariectomized (OVX) rat model is now largely recognized as a suitable osteoporosis
341 preclinical model for the assessment of mandibular bone microarchitecture ⁴⁵. In the rat
342 mandible, the effects of OVX (estrogen depletion) on the alterations of the
343 microarchitectural parameters in the alveolar bone and the condyle have been studied with
344 various experimental conditions that make the interpretation of the existing data complex
345 and incomplete, including the use of different rat strains, different areas of analysis in the
346 mandible, different OVX delays and time post-OVX analyses. These studies also report
347 contradictory data, making the analysis of the effects of aging, on the one hand, and OVX, on
348 the other hand, unclear in the mandible ⁴⁶⁻⁴⁹. With the use of our 3D micro-CT approach
349 allowing alignment of the bone and the BMAT, the present study reports a comprehensive
350 investigation in adult rats that simultaneously studied changes in the bone
351 microarchitectural and BMAT content and its spatial distribution in the alveolar bone and
352 the condyle in a controlled-diet condition, with a clear distinction of age and OVX status.
353 Here, we show that once the rat skeleton is mature ¹⁰ (at 15 months of age), (i) aging does
354 not impact the trabecular bone microarchitecture in both mandibular sites (the alveolar
355 bone and condyle) nor in the tibiae in rats and (ii) OVX has a distinct skeletal impact not only
356 on the mandibular bone microarchitecture but also on the mandibular BMAT content (4-6-
357 fold lower compared to that of the metaphyseal tibial area) and spatial distribution (BMAT
358 remained at a further distance from the trabecular bone surface compared to that of tibia).

359

360 The absence of microarchitectural differences may be due to the advanced age reached by
361 the animals at baseline (6 months) and by a slower remodeling rate that has been reported
362 with advanced age. These hypotheses are consistent with a study of Kribbs *et al.* showing
363 that the decrease in the mandibular bone mass was not correlated with age ⁵⁰. Although
364 some authors reported a lower proliferative capacity and a lower osteogenic differentiation
365 of skeletal stem cells (SSCs) with age ⁵¹⁻⁵³, other data indicate that there are no changes in
366 the differentiation capacities of SSCs with age in rats ⁵⁴, suggesting that the effect of aging
367 may not be synchronized through the whole skeleton. Previous data show that the
368 remodeling activity becomes predominant in rat trabecular bone areas after 6 months of
369 age, suggesting that the skeletal dynamics of rodents may be more relevant to those in the

370 adult human situation than it was previously thought ^{55,56}. Indeed, it has been shown that,
371 similar to higher mammals, the prevailing activity in the tibial trabecular bone of aged (12-
372 month-old) rats is remodeling and not modeling ⁵⁷.

373

374 We also show that, in rats of 10 months of age, OVX-induced mandibular bone loss was
375 three-fold lower than the proximal metaphyseal bone loss in the tibia and was observed only
376 in the alveolar bone (not in the condyle). In Wronski's work, osteopenia was detected in OVX
377 rats as early as 14-days post-OVX in the tibia and became progressively more pronounced up
378 to 100 days ². Afterwards, the tibial trabecular bone volume appeared to stabilize (decreased
379 bone loss and bone formation/resorption indices that were only marginally enhanced). In
380 the present study, no OVX-induced microarchitectural changes were observed between M4
381 and M9; hence, we mainly observed an "early" and "fast" reaction of the body to the surgery
382 (estrogen deficiency) in rats ^{58,59}. In addition, consistent with previous findings, our data
383 show that mandibular bone loss is significantly less important than that in the proximal tibia
384 in response to OVX ^{6,8,60}, suggesting that oral functions may induce stress on mandibular
385 bone cells and prevent oral bone loss. Interestingly, OVX also induced a smaller Tb.N and
386 Tb.Th decrease in both mandibular sites compared to that in the tibia. This small mandibular
387 response to OVX in rodents can be partially explained by the dense trabecular bone network
388 (at baseline) in the mandible ¹². The small response may also be attributed to the unique
389 skeletal characteristics of the mandible, both morphologically and functionally (with the
390 presence of masticatory activity and a 10-fold higher remodeling activity in the alveolar and
391 condylar regions compared to that in the tibia ^{61,62}) and embryologically (with the profile of
392 mandibular skeletal stem cells described as being preferentially osteogenic ^{52,63}). In the
393 present study, transitory hyperphagia was reported after OVX, but it did not induce any
394 changes in the bone microarchitectural parameters in rats, which is in accordance with
395 Jiang's findings, who also reported that there was no impact on the bone strength ⁶⁴. Bearing
396 in mind that the mandible does not represent a typical fracture site in the skeleton, these
397 observations could explain its resistant behavior even in an estrogen-depletion context.

398

399 In addition to the specific mandibular response to OVX, we reported distinct changes in the
400 BMAT content and spatial distribution in the alveolar bone and condyle sites during OVX
401 compared to that in the proximal tibial metaphysis. Indeed, the mandible exhibited a

402 delayed increase in the BMAT content compared to that in the tibia, with a 4-6-fold lower
403 BMAT content, suggesting a slower BMAT conversion rate in the mandible than in the tibia.
404 Interestingly, an alveolar BMAT increase was observed only in the pair-feeding OVX groups,
405 highlighting, for the first time, a protective effect of oral functions on the alveolar BMAT
406 conversion. No age-related BMAT differences were observed at either mandibular site. In
407 the tibia, age-related changes were observed and we believe that the 30% decrease in BMAT
408 content at M9 compared to that at baseline may be due to the diaphyseal growth in the
409 proximal direction that crosses the volume of interest (mainly hematopoietic bone marrow
410 in the central trabecular areas) in aged animals. With the use of our 3D micro-CT approach
411 that allowed us to align the bone and the BMAT ¹², the present study highlights the features
412 of the alveolar and condylar BMAT content and their spatial distribution after OVX in a rat
413 model. Indeed, the spatial distribution of the mandibular BMAT was homogeneously
414 distributed in the first 100 µm from the trabecular bone surface, whereas it was clearly
415 preferentially distributed close to the trabecular bone surface in the tibia (87% of the
416 trabecular bone surface covered by bone marrow adipocytes in the tibia vs approximately
417 5% in both mandibular sites). Bearing in mind the potential role of BMAT in bone
418 metabolism, the distinct metabolic function of the mesenchymal progenitors that are
419 located close to the bone surface and the bone formation areas (the superior proliferative
420 ability and reduced expression of cell cycle inhibitors) reported by Siclari *et al.* may explain
421 the differences in the BMAT content measured in the different skeletal locations in the
422 present study and could provide support for a more metabolically active role of the BMADs
423 in the tibia ³⁴. Our data highlight the importance of investigating not only the BMAT content
424 but also the BMAT spatial distribution in relation to the bone surface and support the
425 hypothesis of a potential role of the BMADs in bone metabolism.

426

427 The micro-CT variables provide biological information that demonstrates the effect of OVX,
428 as shown by PCA (Fig. 6 A-B). These results show that the SHAM and OVX groups do not
429 share the same microarchitectural characteristics. The OVX groups were characterized by a
430 high BMAT content (Ad.V/Ma.V), larger trabecular thickness and trabecular separation as
431 well as a more disconnected trabecular network compared to those of the SHAM group. The
432 SHAM group was characterized by a high trabecular bone volume and number compared to

433 those of the OVX group. Interestingly, bivariate statistical analyses (linear regression)
434 showed only correlations in the OVX groups in the tibia between the bone
435 microarchitectural and BMAT parameters. The negative correlation that was found between
436 the Ad.V/Ma.V and Tb.Th may suggest the involvement of the BMAT content in the
437 trabecular bone thinning in an OVX context. In addition, the positive correlation between the
438 Ad.V/Ma.V and BS/BV has been reported as an excellent predictor of bone loss. No
439 correlations were found in the OVX groups at the mandibular sites, suggesting that BMAT
440 has a smaller impact on trabecular bone loss than that at other sites. The differences in the
441 BMAT content, which were only observed after OVX in the pair-fed group, demonstrate a
442 probable protective role of oral functions in alveolar BMAT conversion.

443 The investigation and understanding of the relationship between BMAT and the bone quality
444 has emerged as an exciting challenge in research in recent years (70-85% of the trabecular
445 bone surface is in contact with the bone marrow). It now appears evident that BMAT should
446 be kept in mind and considered when assessing the bone quality parameters ⁶⁵. By
447 measuring the bone and BMAT within the same region of interest, our data highlight the
448 specific response of the mandibular microarchitecture, BMAT content and spatial
449 distribution during OVX. The distribution pattern of BMAT in the three skeletal locations
450 demonstrates the importance of local factors in the regulation of bone metabolism, in
451 particular BMAT. It would be of great interest to precise the impact of these BMAT changes
452 on the bone quality at the tissue level.

453 In summary, OVX was characterized by marked microarchitectural and BMAT changes in the
454 tibia with significant correlation between the BMAT content and trabecular bone thickness
455 as well as clustering of BMAT close to the trabecular bone surface. By contrast, OVX was
456 characterized by few microarchitectural and BMAT content changes in both mandibular
457 sites, without any correlations between microarchitectural and BMAT variables. According to
458 these data, oral functions may have a protective effect on the BMAT conversion in the
459 alveolar bone in a context of estrogen depletion. These highlights underline the importance
460 of clarifying the long-term effects of local factors, such as the specific BMAT distribution on
461 the mandibular bone quality at the molecular level.

462

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469

470 **CONFLICT OF INTERESTS STATEMENT:** none

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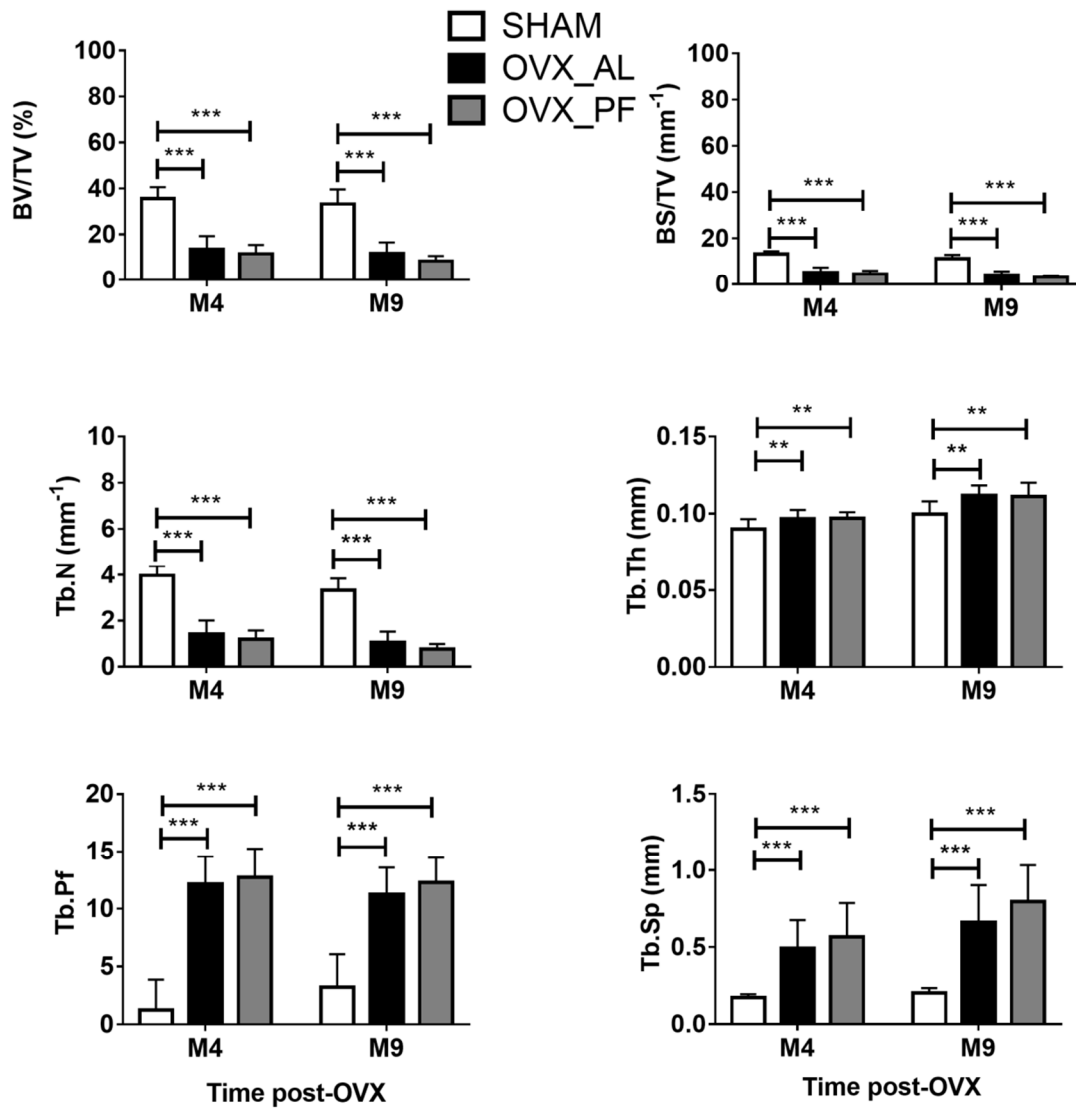
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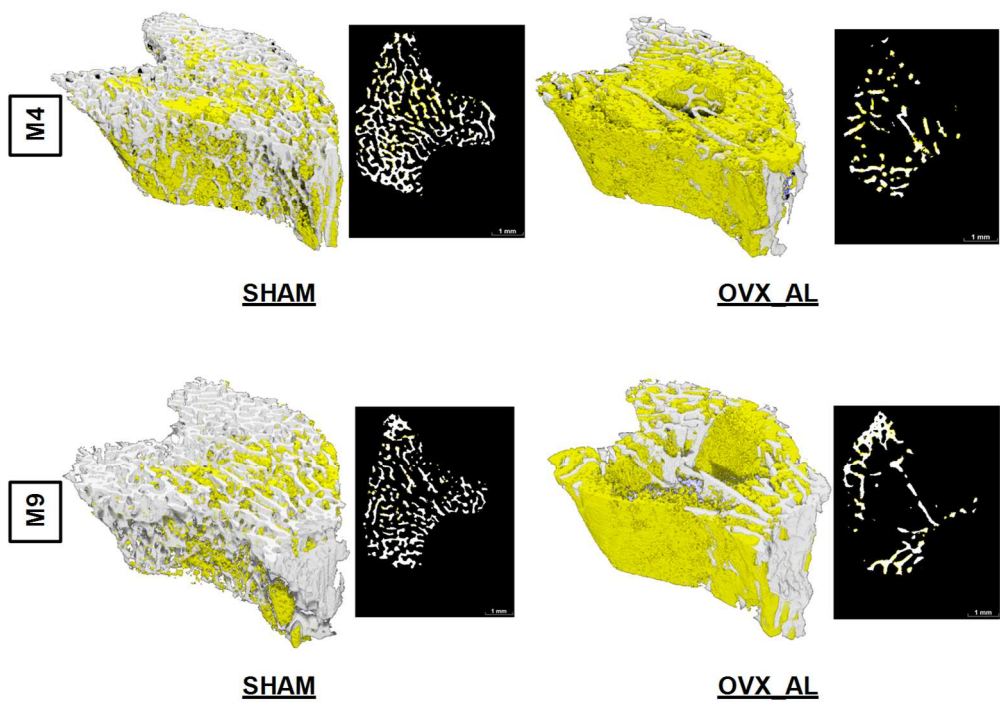
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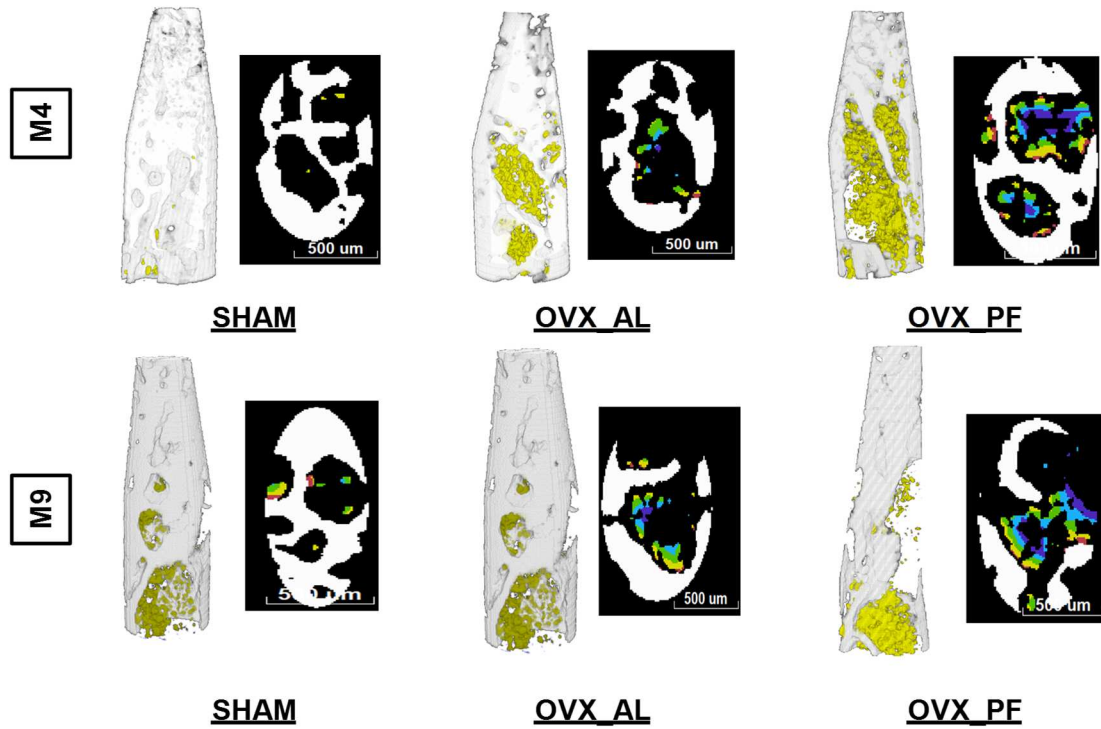
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624 **Figure 8 : OVX-related effect on trabecular bone microarchitecture respectively at M4 and**
 625 **M9 in the tibia. Values are means ± SD. Differences between groups are shown: **p<0.01,**
 626 ***** p<0.0001**



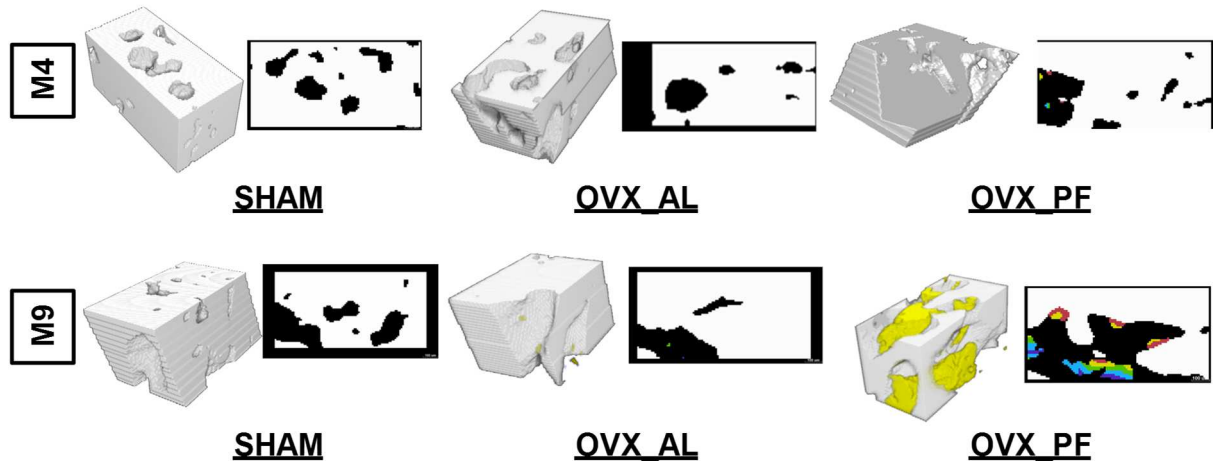
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628 **Figure 9 : 3D micro-CT rendering of trabecular bone microarchitecture (white) and BMAT**
 629 **(yellow) in the proximal metaphysis of the tibia at M4 (top) and M9 (bottom) in SHAM**
 630 **groups (left) and OVX_AL (right). Each volume representation is associated with a 2D**
 631 **transverse cross-section illustrating in 2D the BMAT in contact with the trabecular bone**
 632 **surface.**



633

634 Figure 10 : 3D micro-CT rendering of trabecular bone microarchitecture (white) and BMAT
 635 (yellow) in the alveolar bone at M4 (top) and M9 (bottom) in SHAM (left) , OVX_AL
 636 (middle) and OVX_PF groups (right). Each volume representation is associated with a 2D
 637 transverse cross-section illustrating the distribution of BMAT in relation to the trabecular
 638 bone surface (red: 0-20µm, yellow: 20-40µm, green: 40-60µm, blue: 60-80µm, purple: 80-
 639 100µm)



640

641 Figure 11 : 3D micro-CT rendering of trabecular bone microarchitecture (white) and BMAT
 642 (yellow) in the condyle at M4 (top) and M9 (bottom) in SHAM (left), OVX_AL (middle) and
 643 OVX_PF groups (right). Each volume representation is associated with a 2D transverse
 644 cross-section illustrating the BMAT distribution in relation to the condylar bone surface
 645 (red: 0-20µm, yellow: 20-40µm, green: 40-60µm, blue: 60-80µm, purple: 80-100µm)