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► **To cite this version:**

D. Farlay, Y. Bala, S. Rizzo, S. Bare, J.M. Lappe, et al. Bone remodeling and bone matrix quality before and after menopause in healthy women. BONE, 2019, 128, pp.115030 -. 10.1016/j.bone.2019.08.003 . hal-03487742

HAL Id: hal-03487742

<https://hal.science/hal-03487742>

Submitted on 20 Jul 2022

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1 **Bone remodeling and bone matrix quality before and after**
2 **menopause in healthy women**

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37 **Abstract**

38 Acceleration of remodeling activity after menopause leads to bone loss and fragility, however,
39 whether this is associated with modifications of bone matrix quality has been less studied. The
40 impact of variation in bone remodeling rate on bone matrix has been studied mainly in
41 pathologies or anti-osteoporotic treatments. However, in healthy women this has been less
42 studied. We analyzed, **at the global level**, bone matrix quality in bone biopsies from 3 groups
43 of healthy women (20 per group): 1) before menopause (PreM), 2) 1 year after menopause
44 (PostM, paired biopsies with preM), and 3) 14 (+/-9) years after menopause (LT-PostM). The
45 mean degree of mineralization (DMB) and heterogeneity index (HI) of mineralization were
46 assessed by X-ray microradiography **on whole bone matrix**; **i**ntrinsic properties
47 (mineral/organic ratio, mineral maturity, mineral crystallinity, collagen maturity) were
48 assessed by Fourier Transform Infrared microspectroscopy, microhardness by
49 microindentation, **both at a global level and calculated by mean of several measurements over**
50 **the whole tissue area**. In PostM compared to PreM (bone remodeling rate had doubled), mean
51 DMB measured on the entire bone plane (**whole bone matrix**) of the sample was not different.
52 HI was increased in trabecular bone indicating a higher heterogeneity of mineralization.
53 However, in PostM, mineral/organic ratio (trabecular) and microhardness (cortical and
54 trabecular) were decreased, whereas mineral/collagen maturation or crystal size/perfection
55 were unchanged. Thus, in PostM, the **local** mineral content and microhardness were first
56 affected. In LT-PostM (bone remodeling rate was 3 times higher), the mean DMB was still
57 not different. However, the mineral/organic ratio, microhardness, mineral maturity,
58 crystallinity all were lower compared to PreM and PostM, in both cortical and trabecular
59 bone. Bone remodeling rate was negatively correlated with microhardness, DMB,
60 mineral/organic and crystallinity. This suggests that increases in bone remodeling rates after
61 menopause have a direct impact on bone quality by inducing the formation of more extensive

62 “immature” bone areas, but the amount of immature bone does not cause modification of the
63 global DMB.

64 **Key words**

65 Menopause, bone remodeling, degree of mineralization, microhardness, intrinsic material
66 properties.

67

68
69 **Highlights**

70

- 71 • After menopause, bone loss is associated with increased bone remodeling, but the
72 change in bone matrix quality is less well-known.
- 73 • Bone matrix quality was analyzed on biopsies from healthy women before (PreM), 1
74 (PostM) and 14+/-9 years after menopause (LT-PostM).
- 75 • Compared to PreM, the degree of mineralization of bone was unchanged in PostM and
76 LT-PostM despite increased bone turnover.
- 77 • In PostM, decreases in mineral content (mineral/organic ratio) and microhardness
78 were detected.
- 79 • In LT-PostM, these changes were confirmed, and were associated with decreased
80 mineral maturity and crystallinity.

81

82 **Introduction**

83

84 Menopause increases bone fragility in women, in part by decreasing bone mass and
85 bone mineral density (BMD). [1] The association of oestrogen deficiency with bone loss and
86 accelerated bone remodeling is well-established. Oestrogen deficiency is associated with an
87 unbalanced coupling between resorption and formation in favour of bone resorption,
88 gradually leading to bone loss. [1][2][3] Recker et al have shown by histomorphometry that
89 the activation frequency (Ac.f) doubled 1 year after menopause (paired biopsies), and tripled
90 after 13 years. [4] The increase in bone turnover impacts bone mass and microarchitecture
91 (both standard 2D histomorphometry and 3D micro-CT measurements) by deteriorated
92 trabecular bone structure. [5] However, the effect of the increase in remodeling after
93 menopause on bone quality is less known, especially on the bone matrix which is one of the
94 determinants of bone strength. [6] Bone matrix quality reflects the intrinsic traits of the
95 matrix, for example its degree of mineralization, the apatite mineral crystals characteristics,
96 organic matrix characteristics and more. Mineralization of bone consists of 2 steps, the first
97 one named “primary mineralization” is a rapid initial deposition of mineral in organic matrix,
98 and the second one named “secondary mineralization” continues until the upper mineral
99 density is reached in a given volume of matrix, and is associated with a slow maturation of
100 apatite crystals. [7] [8] [9] This results in the emergence of bone structural units (BSUs)
101 formed during a remodeling cycle, with new BSUs characterized by low mineralization, and
102 old interstitial BSUs by high mineralization. [9] This leads to the formation of numerous
103 osteons with different mineralizations. Both degree of mineralization and its heterogeneity are
104 important in terms of bone strength, as bone mineral impacts the stiffness of bone, and its
105 heterogeneity inhibits crack propagation. The level of bone turnover influences the degree of
106 mineralization of bone, as observed in some pathologies (hyperparathyroidism with

107 hypercalcemia), or after anti-osteoporotic treatments (teriparatide, estrogen, SERMS,
108 bisphosphonate, denosumab), [10] [11] [12] [13] [14] [15] but the influence of physiological
109 remodeling on bone matrix, in the absence of treatment has less been studied. On 17 paired
110 biopsies before and 1 year after menopause, no difference was found in bone mineralization
111 density distribution (BMDD) or in the heterogeneity index (CaWidth) by quantitative
112 backscattered electron imaging (QB EI). [16] Thus, it has been suggested that bone
113 mineralization changes are delayed, but no study has been performed to evaluate the degree of
114 mineralization and intrinsic properties after long-term menopause in healthy women. [16]

115 The hypothesis of the present study is that the sustained increase in bone turnover after
116 long-term menopause is associated with decreases in bone mineralization and microhardness,
117 and modifies the characteristics of bone matrix (mineral maturity, crystallinity, collagen
118 maturity). Thus, the purpose of the present study was to evaluate the bone mineralization and
119 the bone matrix characteristics in transiliac bone biopsies obtained in healthy untreated
120 women before menopause, 1 year after menopause, and 14 years after menopause. The degree
121 of mineralization of bone tissue, and its heterogeneity index were assessed by digitized
122 microradiography [17], the bone material intrinsic properties reflected by mineral/organic
123 ratio, mineral maturity, crystallinity and collagen maturity [18] by Fourier Transform Infrared
124 microspectroscopy (FTIRM), and biomechanical behavior by microindentation. [19]

125

126 **Material and Methods**

127 Bone specimens:

128 Bone samples have been previously analyzed by bone histomorphometry. [4] Paired transiliac
129 bone biopsies were obtained from 20 healthy women when they were pre-menopausal (49 ± 2
130 years) (PreM group) and 1 year after the last menstrual period (55 ± 2 years) (post-
131 menopausal, PostM group). Time at menopause was defined by the date of the last menses.

132 Levels of serum estradiol at the time of the second biopsy were $\leq 20\text{pg/ml}$ and FSH > 75
133 mIU/ml assessed during the follow-up of the subjects. Another group of 20 biopsies were
134 collected in healthy postmenopausal women (60 ± 7 years) 14 ± 9 years after menopause
135 (LONG-TERM POST, LT-PostM group). None of the patients included in this study had ever
136 had a fracture prior to, or during, observation in the group followed pre-and post-menopause
137 (PreM-PostM). Also, none of the LT-PostM subjects had ever suffered a fracture prior to
138 enrollment. The PreM-PostM, and the LT-PostM subjects were screened prior to enrollment
139 in their respective studies. The screening included careful histories, physical exams, and
140 clinical laboratory exams to rule out any disease or diagnosis occurring prior to enrollment.
141 The entry criteria are described in the prior publications [4] [20] [21]. All were Caucasian and
142 were screened for good health, absence of any fracture, diagnosis or abnormal condition. The
143 LT-PostM group were all Caucasians, and between 5 and 23 years past menopause when
144 biopsied. Bone specimens were embedded in methyl methacrylate after fixation and
145 dehydration in alcohol.

146 One hundred $\pm 1\mu\text{m}$ -thick sections were cut and polished with a diamond paste ($1\mu\text{m}$) for the
147 measurement of microhardness by microindentation. Sections were then ground into $50 \pm$
148 $1\mu\text{m}$ -thick sections used for measurement of the degree of mineralization by digitized
149 microradiography. Two- μm thick sections were cut from embedded blocks for the analysis of
150 mineral and organic characteristics by FTIRM analysis.

151

152 *Histomorphometry*

153 Histomorphometric analysis of the three groups has been described elsewhere. [4] Static and
154 dynamic variables were measured or calculated. Trabecular bone volume/total volume
155 (BV/TV, %), trabecular thickness (Tb.Th, μm), activation frequency (Ac.f, #/year), bone
156 formation rate surface referent (BFR/BS, $\mu\text{m}^3/\mu\text{m}^2/\text{year}$), **bone formation rate volume referent**

157 (BFR/BV, %/year) osteoclast surface (Oc.S/BS, %), mineralizing surface (MS/BS, %), wall
158 thickness (W.Th, μm) and formation period (FP, year) have been collected from the co-
159 authors' database, and methodology has been published elsewhere. [22] Variables of bone
160 structure included BV/TV, Tb.Th and W.Th. Variables of bone remodeling included Oc.S/BS,
161 Ac.f, BFR/BS, BFR/BV, MS/BS and FP.

162

163 *Digitized Microradiography:*

164 The degree of mineralization of bone (DMB) was measured by the technique of digitized
165 microradiography. [17] Briefly, both 100- μm and 50- μm thick bone sections were analyzed
166 with a tube Microfocus Hammamatsu X-ray system L9421-02 with power maxi 8 W, a
167 copper anode, a nickel filter, a Beryllium window of 150 μm and a focal spot size of 5 μm in
168 diameter. The exposure parameters are high voltage: 40 kV, current: 50 μA and power of 2
169 W. The source to object distance was in a range of 1–25 cm and the object to detector distance
170 was also in the range of 1–25 cm. The detector was a Photonic science CCD camera FDI
171 VHR 11 M with an active area of 36 \times 24 mm (4008 \times 2671 pixels). The scintillator was
172 Gd₂O₂S: Tb, and an aluminum filter of 12 μm was used. The image digitization step was
173 done with a 12-bit digital image detector (pixel size: 9 μm , object pixel size: 0.83 μm).
174 Thresholds of 0.6 g/cm³ and 0.3 g/cm³ were used for the 100- μm and 50 μm -thick sections,
175 respectively. A reference aluminium step-wedge was exposed on each Geola high-resolution
176 film for 20 min (Slavich International Wholesale Office, Vilnius, Lithuania) when bone
177 sections were measured for the quantitative evaluation of X-ray absorption. The mean DMB
178 and mean heterogeneity index (HI = full width at half maximum of the curve of distribution)
179 of the DMB were expressed in g mineral/cm³ and measured in trabecular and cortical bone
180 separately.

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Fourier Transform InfraRed Microspectroscopy (FTIRM) analysis

FTIRM allows assessment in situ of physicochemical modifications of bone matrix, identified by the vibrational response of different constituents, with the main advantage to measure simultaneously, the mineral (phosphate and carbonates) and organic matrix (amide I, II and III).

Analysis of bone material intrinsic properties was performed on cortical and trabecular bone separately, according techniques previously described. [23] [24] Briefly, thin bone sections (2- μm thick) were analyzed in transmission mode with a Perkin-Elmer GXII Auto-image Microscope (Norwalk, CT, USA) equipped with a wide band detector (mercury-cadmium-telluride) ($7800\text{-}400\text{ cm}^{-1}$). A Cassegrain objective with numerical aperture of 0.6 was used with a spatial resolution of $10\text{ }\mu\text{m}$ at typical mid-infrared wavelengths. Ten areas ($150\mu\text{m}\times 150\text{ }\mu\text{m}$) in each cortex, and 20 areas ($150\times 50\mu\text{m}$) in trabecular bone were scanned, and randomly chosen. After curve-fitting of infrared spectra, 4 variables were measured: mineral maturity, crystallinity, mineral/organic ratio, and collagen maturity. Each spectrum was collected at 4 cm^{-1} resolution, and 50 scans by spectrum were performed in the transmission mode. Contributions of air and PMMA were subtracted from the original spectrum. After automatic baseline correction (Spectrum Software) and curve fitting of every individual spectrum, GRAMS/AI software (Thermo Galactic, Salem, NH, USA) was used to quantify the characteristics of the spectra. The following parameters were determined: the mineral crystallinity (cryst) which is inversely proportional to the full width at half-maximum of the 604 cm^{-1} peak (apatitic phosphate environment) and corresponds to both crystal size and perfection, [23] the mineral to organic ratio (min/org) i.e. the area ratio of the bands of mineral matrix over organic matrix ($1184\text{-}910\text{ cm}^{-1}/1712\text{-}1592\text{ cm}^{-1}$), [25] the mineral maturity (min mat) which is calculated as the area ratio of the apatitic phosphate over non

206 apatitic phosphate (1030/1110 cm^{-1} area ratio) and reflects the age of mineral, [23] the
207 mineral crystallinity ($\text{cryst} = 1/\text{FWHM}$ of the 604 cm^{-1} peak, [23]), and the collagen maturity
208 (coll mat) which is calculated as the ratio of organic matrix bands (1660/1690 cm^{-1} area ratio).
209 [24] [26] While this ratio has been shown to be related to collagen enzymatic cross-links, we
210 did not manage to show a relationship between the ratio 1660/1690 cm^{-1} and enzymatic cross
211 links biochemically measured. [24] [26] However, in untreated human bone, we observed
212 correlations between this ratio and the mineral maturation, suggesting that an increase in this
213 ratio could be related to a modification of the collagen secondary structure related to the
214 mineralization process. Results are expressed as mean \pm standard deviation (SD) for each
215 bone envelope (cortical or trabecular).

216

217 *Microhardness testing:*

218 Hardness measurements reflect the resistance to material deformation, which is the ability of a
219 material's surface to resist indentation. Microhardness was measured on the same bone
220 sections used for microradiography, using a Micromet 5104 (Buehler, Lake Bluff, Illinois,
221 USA) equipped with a Vickers indenter. [27] Briefly, microhardness tests were performed
222 using a test load of 25 g applied for 10 seconds. For each sample, 40 indents (20 in cortical
223 and 20 in trabecular bone) were performed at the tissue level, and randomly chosen.
224 Indentations were performed in randomly selected areas of the bone surface, separated by at
225 least 500 μm over the whole bone tissue area.

226 Microhardness (Hv) was derived from the direct measurement of diagonal dimensions using
227 manufacturer software (Omnimet HMS v.2.31), and using the following formulae $Hv = 1854.4$
228 P/d^2 (where Hv is Vickers microhardness expressed in kg/mm^2 , P is test load in kg and d is
229 the mean length of the two diagonals expressed in mm). Results are expressed as mean \pm
230 standard deviation (SD) for each bone envelope (cortical or trabecular).

231 ***Statistical analyses:***

232 Analyses were performed using SPSS® 16.0 (SPSS Inc., Chicago, IL). Differences between
233 paired biopsies were assessed using non-parametric Wilcoxon tests. Differences between the
234 LT-PostM group and the PreM or PostM groups were assessed using non-parametric Mann-
235 Whitney tests. The relationships between DMB, mineral and collagen characteristics, local
236 mechanical properties and remodeling parameters were tested using spearman correlations (r')
237 and illustrated by a HeatMap performed on RStudio-software (v 1.1.463). A p-value < 0.05
238 was used to define statistical significance.

239

240 **Results**

241 ***Histomorphometric analysis*** BV/TV and Tb.Th were not different between the 3 groups
242 (Table 1). When compared to the PreM group, PostM and LT-PostM groups were
243 characterized by a significantly higher Oc.S/BS ($p=0.003$ and $p=0.001$ in PostM and LT-
244 PostM, respectively, Table 1). It was associated with significantly higher values in parameters
245 reflecting bone remodeling ($p<0.05$ to 0.002) and a shorter duration of the formation period
246 ($p=0.003$ and $p=0.01$ in PostM and LT-PostM, respectively, Table 1). No significant
247 difference was shown between the PostM and LT-Post group. W.Th did not change in the
248 PostM group compared to PreM but was significantly lower in the LT-Post group when
249 compared to the two other groups.

250

251 ***Degree of mineralization (DMB, Fig. 1A)***

252 In PostM and LT-Post groups, the DMB was not different in either cortical or trabecular bone
253 compared to the PreM group (Fig. 1B). An increase in trabecular HI in the PostM compared
254 to the PreM group ($p=0.033$), and decreases in cortical HI in the LT-Post group compared to
255 the PreM ($p=0.028$) and PostM ($p=0.008$) groups were observed (Fig. 1C).

256

257 ***Mineral and organic characteristics (Fig. 2A)***

258 In paired biopsies, the only difference was a decrease in min/org ratio in trabecular bone after
259 one year of menopause [-3.11%, p=0.024 PostM vs. PreM (Fig. 1B)]. No difference was
260 observed in cortical bone.

261 In LT-Post group, min/org ratio, min mat and cryst were lower than in PreM in both cortical (-
262 8 %, p<0.0001; -10.16%, p=0.012; -2.55%, p=0.019 respectively) and trabecular bone (-
263 8.49%, p=0.0007; -8.54%, p=0.02; -3.35%, p=0.021, respectively (Fig. 2B-C-D). Coll mat
264 was unchanged (Fig. 2E). Compared to the PostM group, LT-Post group showed a lower
265 min/org and min mat ratios in both cortical and trabecular bone, and a lower cryst in
266 trabecular bone (Fig. 2 B-C-D). Coll mat was unchanged (Fig. 2E).

267

268 ***Microhardness (Fig. 3A)***

269 In PostM group, the microhardness was significantly decreased by 4.4 % (p<0.044) and 4.1 %
270 (p=0.040) in cortical and trabecular bone, respectively, compared to PreM (Fig. 3B). In the
271 LT-PostM group, microhardness was highly significantly lower than in PreM and PostM
272 groups, reaching -8.74% in cortical bone (p=0.0002) and -13.32% in trabecular bone
273 (p<0.0001), respectively (Fig. 3B). Compared to PostM, LT- PostM group was significantly
274 lower in trabecular bone only (p=0.0001).

275

276 ***Correlations with histomorphometric variables of bone remodeling and DMB,
277 microhardness values, and mineral/organic characteristics***

278 Table 2 summarizes the expected effects of increase in bone remodeling (Ac.f), and the
279 observed effects on bone matrix quality in trabecular bone. As variables of bone remodeling
280 were not measured in cortical bone, we analyzed only the correlations with trabecular bone

281 quality variables. Several variables of bone remodeling were correlated with trabecular bone
282 matrix quality. Ac.f and BFR/BS were negatively correlated with trabecular DMB, trabecular
283 cryst, trabecular min/org, and trabecular microhardness, (Fig. 4). MS/BS was negatively
284 correlated with trabecular min/org ratio and trabecular microhardness. Osteoclast surface
285 (Oc.S/BS) was negatively correlated, and the formation period (FP), was positively correlated
286 with microhardness (Fig. 4). To illustrate the dispersion of points, both correlations (between
287 Ac.f and trabecular min/org and microhardness) with split groups were shown in
288 supplementary files (Fig.1S).

289

290 Discussion

291 The aim of this study was to assess the impact of the increase in bone remodeling after
292 menopause on bone matrix quality. Sub-groups used in the present study and the
293 histomorphometric results come from a larger population previously published by Recker et
294 al. [4] [21] The bone turnover (Ac.f) was twice and 2.5 times higher in the PostM and the LT-
295 PostM respectively compared to PreM group, without any significant difference between
296 PostM and LT-PostM groups. Oc.S/BS and MS/BS were also higher in both PostM and LT-
297 PostM groups, confirming the marked increase in bone remodeling and consequently the
298 presence of more new remodelling sites after menopause. W.Th and FP were lower in the LT-
299 PostM group compared to both the PreM and PostM groups, showing a lower amount of bone
300 formed at each bone remodeling unit, but BV/TV and Tb.Th were not significantly decreased
301 at that time-point. We showed that, at one year after menopause, in paired biopsies, when
302 bone remodeling rate is doubled, the mean DMB of the total bone tissue was unchanged. An
303 increase in HI was observed in trabecular bone, reflecting the formation of new BSUs, but the
304 occurrence of new remodeling sites was not sufficient to modify the mean DMB. In contrast,
305 when measured in randomly selected bone areas, the min/org ratio in trabecular bone, and

306 microhardness in both cortical and trabecular bone decreased. Furthermore, mineral/collagen
307 maturity and crystal/size perfection were not modified, suggesting that, even if **locally**,
308 secondary mineralization was not completed, the organic matrix had sufficient time to fully
309 mature, and the deposited apatite crystals to grow. After 14 (+/-9) years of menopause
310 (unpaired group), despite an almost triple increase in bone remodeling, the mean DMB was
311 identical. It was associated, **locally**, in both cortical and trabecular bone, with min/org ratio,
312 min mat, cryst and microhardness becoming lower than in both pre and post menopause. This
313 could indicate that, in addition to not being fully mineralized, the new remodeling units
314 contained apatite crystals which were not yet fully mature and had not reach their final size.

315 **As the skeleton is completely renewed every 10 to 15 years, it can be assumed that a**
316 **large proportion of the skeleton has been remodeled in the LT-PostM group since the date of**
317 **menopause**, and consequently, a diminution of DMB **was expected**. In contrast, our present
318 results showed no change in both cortical and **trabecular** DMB in the two post-menopausal
319 groups, but the lower min/org ratio suggested the presence of not totally mature crystals. **The**
320 **main differences between the two different ways for the assessment of the mineral content**
321 **were: 1) the DMB (X-ray microradiography) was measured on the whole tissue area, taking**
322 **into account the amount of interstitial and newly formed bone. The DMB expressed in g/cm³**
323 **mineral is an absolute value (density of mineral per unit volume of bone) and 2) the min/org**
324 **ratio (FTIRM) which is a relative ratio between phosphate/amide and measured locally**
325 **(several area of measurements randomly selected over the whole tissue area). The DMB**
326 **(g/cm³) is preferred to min/org ratio for the measurement of bone mineral content, however,**
327 **the advantage of FTIRM is that several variables can be simultaneously measured, thus giving**
328 **additional informations on mineral and organic characteristics.** One possible explanation for
329 this apparent discrepancy may be an increase in the organic matrix content **in the measure of**
330 **the min/org ratio** but **as** BV/TV did not change, **we looked for another explanation.**

331 Methodological conditions may influence the DMB measurements, especially the thickness of
332 the sections. DMB was also measured on 50 μm -thick sections in order to reduce potential
333 influence of the partial volume, but it was still identical between the 3 groups. Our results
334 were in agreement with a previous study performed in a subgroup of biopsies (n=17/group)
335 from the same study (PreM- PostM pairs only) [16] which reports no change in bone
336 mineralization density distribution (BMDD) measured by quantitative backscatter electron
337 imaging (QBEB). Only a trend toward increase in Ca width was observed. In a study
338 performed by Ciarelli et al. on iliac trabecular bone from patients with either low or high
339 BFR, with or without vertebral fractures, there was no difference in average mineralization,
340 while the standard deviation of the mineralization (reflecting heterogeneity of the
341 mineralization) was higher in high BFR compared to low BFR groups. [28] We also found an
342 increase in HI one year after menopause (PostM) but in the LT-PostM group, HI in cortical
343 bone, was lower than in both the PreM and PostM group, despite the sustained increase in
344 bone remodeling. In LT-PostM, the increase in bone resorption associated with a diminution
345 of W.Th may result in a higher proportion of interstitial bone than recent bone and
346 consequently explained the lower HI and the trend to increase DMB.

347 As mentioned above, we found a discrepancy between DMB and min/org ratio, both
348 from assessing the mineral content. The main difference was the bone surface analyzed. The
349 entire whole matrix is taken into account for DMB measurements, whereas FTIRM and
350 microhardness were performed at local level, and variables calculated by the mean of local
351 measurements over the whole tissue area. We have to keep in mind that the analysis area
352 selection criteria are of paramount importance as intrinsic properties are greatly dependant on
353 tissue age. [29] While randomly performed, it is likely that FTIRM, or microhardness,
354 measurements in recent bone were both favored. Both in cortical or trabecular bone, the
355 ability to measure several young osteons simultaneously (rather than only interstitial bone) is

356 high due to the spatial resolution used ($150 \times 150 \mu\text{m}^2$ in cortical and $50 \times 150 \mu\text{m}^2$ in trabecular
357 bone, see fig 1A). We found that, early after menopause, in the PostM group, the min/org
358 ratio was decreased in trabecular bone (without change in cryst, min mat and coll mat), and
359 microhardness decreased in both trabecular and cortical bone. Thus, the 2-fold higher increase
360 in bone remodeling early after the menopause mainly affected the amount of mineral
361 deposited in recent BSUs. However, the increased bone remodeling did not disturb the
362 mineral maturity and crystallinity of apatite crystals. In LT-PostM, the fact that values of
363 cortical and trabecular min/org ratio, min mat, cryst, and microhardness were lower than
364 PostM and PreM, may be explained by a higher number of “younger” BSUs. Thus, the
365 increase in bone turnover results in the accumulation of “immature” mineral characterized by
366 a lower cryst (size/perfection of crystal), min mat (proportion of apatitic versus non-apatitic
367 domains), associated with a lower min/org ratio in both cortical and trabecular bone. We
368 expected a correlation between Ac.f and both min mat and coll mat, however, no such
369 correlations were observed. While coll mat and min mat were well-correlated in the whole
370 population (60 samples), as illustrated on the HeatMap, coll mat was not different in the LT-
371 Post group compared to both PreM and PostM groups, whereas min mat was lower. This
372 suggests a more rapid maturation of collagen than mineral. In a subgroup of 9 biopsies of the
373 initial Recker study, [30] Raman microspectroscopy performed on the active bone trabecular
374 forming surface identified by fluorescent labels (right under the first label on trabeculae,
375 designed as “older”), shows an increase in the min/org ratio explained by a decrease in
376 collagen content without change in mineral content. The discrepancy with our present results
377 could be explained by the number of measurements and the different spatial resolution: 20
378 measurements at a spatial resolution of $50 \times 150 \mu\text{m}^2$ representing $\sim 150\,000 \mu\text{m}^2$ per biopsy in
379 the present study against 3 regions in 3 trabeculae at spatial resolution $< 1 \mu\text{m}^2$ representing \sim
380 $9 \mu\text{m}^2$ in the Raman study[30]. In addition, the number of bone biopsies was lower than in our

381 study (9 compared to 20 biopsies per group), and only PreM and PostM groups were
382 analyzed. Moreover, the site of measurement was different (between 2 fluorescent labels
383 compared to several randomly chosen BSUs). However, we are in agreement with the
384 crystallinity and the ratio reflecting organic matrix, since they also reported no difference in
385 the crystallinity or 1660 cm^{-1} /amide I between PreM and PostM groups as in our study
386 ($1660/1690\text{ cm}^{-1}$ ratio). In another study performed by the same group, originating from
387 several studies performed by Raman microspectroscopy, it was shown that in postmenopausal
388 osteoporotic women the mineral/org ratio was not different from the healthy postmenopausal
389 group except in the region right under the first label where the min/org ratio was lower. [31]
390 No alterations in local mechanical properties (hardness and stiffness) measured by either
391 quasi-static or dynamic nanoindentation were reported on a subset of bone biopsies used in
392 the present study. [32] It is important to note that nanoindentation tests result in indents of a
393 few microns in diameter and therefore reflect mechanical properties of lamellae.
394 Microhardness merges the mechanical response of several lamellae and is therefore not
395 sensitive to variation in the main orientation of collagen fibers between lamellae, and affects
396 measurements independently of the amount of mineral. [33] This also explains the large
397 variation in values obtained by nanoindentation that may have hidden differences between the
398 two groups.

399 Even if the number of new BSU (packets or osteons) was higher after menopause, this
400 was not sufficient to induce a variation of DMB in the entire bone biopsy in LT-PostM. In
401 other words, bone maintains an “optimal” global DMB probably offsetting the bone loss and
402 focal changes in mineral bone matrix, in order to optimize bone strength. This could be
403 modulated by an optimal ratio between new/interstitial bone area, leading to maintenance of a
404 stable DMB. In a study performed on human osteoporotic single trabeculae, Busse et al.
405 identified different patterns of mineralization with high interstitial mineralization and low

406 mineralized area in non-osteoporotic trabeculae, and high mineralized “rod-like” trabeculae
407 with highly mineralized cement lines in several bone packets within similar “rod-like”
408 trabeculae. [34] The mean calcium content was increased in osteoporotic compared to non-
409 osteoporotic trabeculae, due to the accumulation of several “old” BSUs”. Thus, in the present
410 study, despite an increased bone turnover, a sufficient number of “old” BSUs with a high
411 DMB were present in interstitial bone. They contribute to the total DMB measurement more
412 than the “young” BSUs with a low DMB. Thus, we can speculate that, as bone in LT-PostM
413 was “healthy”, despite the fact that a decrease in bone formation begins to appear, a
414 proportion of interstitial, highly mineralized bone, pulls the values of DMB up.

415 Bone microhardness and Young’s modulus are mostly related to the mineral content at the
416 organ level and BSU level [19] [33] [7] [35] [36]. Bala et al. showed that, at the BSU level,
417 contact microhardness (reflecting both elastic and plastic behaviors) was correlated with
418 DMB and collagen maturity [33]. It is important to emphasize that the correlation between
419 mineral content and micromechanical properties are mostly visible when focal measurement
420 at the same level of individual BSUs are performed. As the aim of the present study was to
421 assess the impact of increase in bone remodeling on bone matrix quality variables, we did not
422 measure the bone quality variables at the BSU level but at a global level, taking into account a
423 larger proportion of bone matrix. No modification of DMB between the 3 groups was found,
424 however, we found a decrease in both mineral/organic matrix and microhardness. These two
425 variables, which were measured on randomly selected areas over the total bone tissue,
426 decreased in the same manner. As collagen maturity was not different between groups, our
427 results suggested that the increase in bone remodeling primarily influenced the mineral
428 content. However, we cannot exclude the possibility that other components of the bone matrix
429 might influence bone mechanical properties, such as water [37] and collagen crosslinks. [38]
430 [39] Nevertheless, we found relationships between histomorphometric data and bone

431 material properties, showing how variation in bone remodeling rates could influence bone
432 material properties. Indeed, the more the bone remodeling increases, the lower the
433 min/matrix, the crystal size/perfection and the microhardness are, and the higher the
434 heterogeneity of mineralization is. Despite these facts, we did not find differences between the
435 3 groups for DMB, and we observed a negative correlation between Ac.f. and trab DMB,
436 confirming the influence of the increased remodeling on DMB after menopause. However,
437 due to overlap in the level of remodeling of bone between groups, a significant difference
438 between groups could not be evidenced. In a study comparing mineral quality and quantity
439 assessed by FTIRM in iliac crests from post-menopausal osteoporotic women, with high or
440 low turnover, Boskey et al. showed that the min/org ratio was slightly reduced with high-
441 turnover compared to normal controls, but not different with low turnover. [40] However she
442 found that the crystallinity was significantly increased in both low- and high-turnover cases,
443 whereas in the present study, we found a decrease in crystallinity.

444 The main limitation of this study was that paired biopsies were analyzed in PreM and
445 PostM groups while the LT-PostM menopause was an independent group whose
446 premenopausal level of bone remodeling was unknown. This could explain the lack of
447 differences in DMB between LT-PostM versus PreM and PostM. However, we found lower
448 values than in the two other groups reflecting the intrinsic properties in the LT-PostM. In
449 addition, the time since menopause was heterogeneous (14+/- 9 years). Finally, the number of
450 bone biopsies (20 per group) was low.

451 To conclude, the increase in bone remodeling 1 year after menopause induces the
452 formation of bone with locally, lower mineralization in trabecular bone, without influencing
453 the global degree of mineralization. In long-term post-menopausal healthy women (14 +/-9
454 years after menopause), local values of mineralization are even lower than in women one year
455 after menopause, with a lower mineral maturity, crystallinity and microhardness. However,

456 the global degree of mineralization was not affected. Thus, in healthy long-term post-
457 menopausal women, the mean degree of mineralization was unchanged, but **local** changes in
458 bone matrix intrinsic properties began to appear.

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462 **Acknowledgements**

463 The authors would like to thank Dr P. Chavassieux and JP. Roux for helpful discussion
464 concerning the histomorphometric interpretation of the data, and P. Wagner for his statistical
465 help in HeatMap construction.

466 Authors'roles: Study design: DF, YB, JL, RR, and GB. Study conduct: DF, YB, GB. Data
467 collection: DF, YB, SR, SB. Data analysis: DF, YB, RR and GB. Data interpretation: DF, YB,
468 JL RR GB. Drafting manuscript: DF, YB. Revising manuscript content: DF, YB, SR, SB, JL,
469 RR, GB. Approving final version of manuscript: DF, YB, SR, SB, JL, RR, and GB. DF takes
470 responsibility for the integrity of the data analysis.

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Figures Legends

Figure 1. A-Digitized microradiographs of PreM, PostM and LT-PostM with cortical (Top) and trabecular bone (Bottom). Box-plot representation of B- degree of mineralization (DMB, g/cm^3) and C- heterogeneity Index (g/cm^3) in PreM, PostM and LT-PostM groups. DMB values were not significantly modified in the 3 groups, in cortical and trabecular bone.

Figure 2. Focal measurements of mineral and organic characteristics assessed by Fourier Transform Infrared microspectroscopy. A- Two μm thick section of LT-PostM with examples of area measured in cortical (top) and trabecular bone (bottom). Blox-plot representations of B- Mineral/organic ratio, C- Mineral maturity, D- Crystallinity, E- Collagen maturity. After one year of menopause (PostM), the mineral/organic ratio is decreased in trabecular bone only. After 14 years of menopause (LT- PostM), Crystallinity, mineral maturity, mineral/organic ratio are decreased in both cortical and trabecular bone. Collagen maturity was unchanged.

Figure 3. A. Focal measurements performed by Vicker indentation in cortical (Top) and trabecular (Bottom) bone. B. Box-plot representation for Microhardness assessed in PreM, PostM and LT-PostM. Significant decreases in microhardness are observed in PostM and LT-PostM compared to PreM.

Figure 4. HeatMap illustrating spearman ($\rho=r'$) correlations between age and time of menopause, the histomorphometric, micromechanical and intrinsic variables. Blue squares indicate a positive correlation and red squares a negative correlation. Square color indicates the intensity of the correlation showed in the colored bar. A darker blue or red square corresponds to the best positive (blue) or negative (red) correlation. Significance is mentioned by stars within the squares. * $p<0.05$ ** <0.01 *** $p<0.001$. The absence of stars indicates the absence of significativity.

Supplementary file:

Figure 1S. Spearman correlations between A. Ac.f (histomorphometry) and Trabecular min/org ratio (FTIRM) B. Ac.f (histomorphometry) and Trabecular microhardness (Vicker microindentation) in PreM, PostM and LT-PostM groups. The more the bone remodeling increases (Ac.F), the lower the mineral content (trab min/org ratio) and the microhardness are.

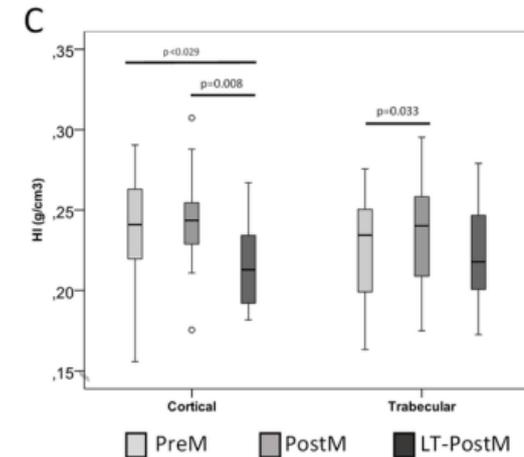
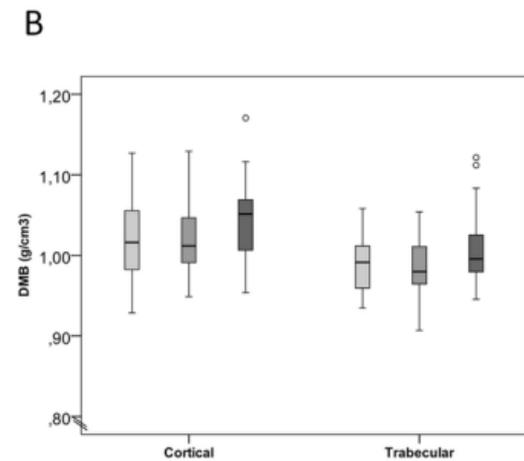
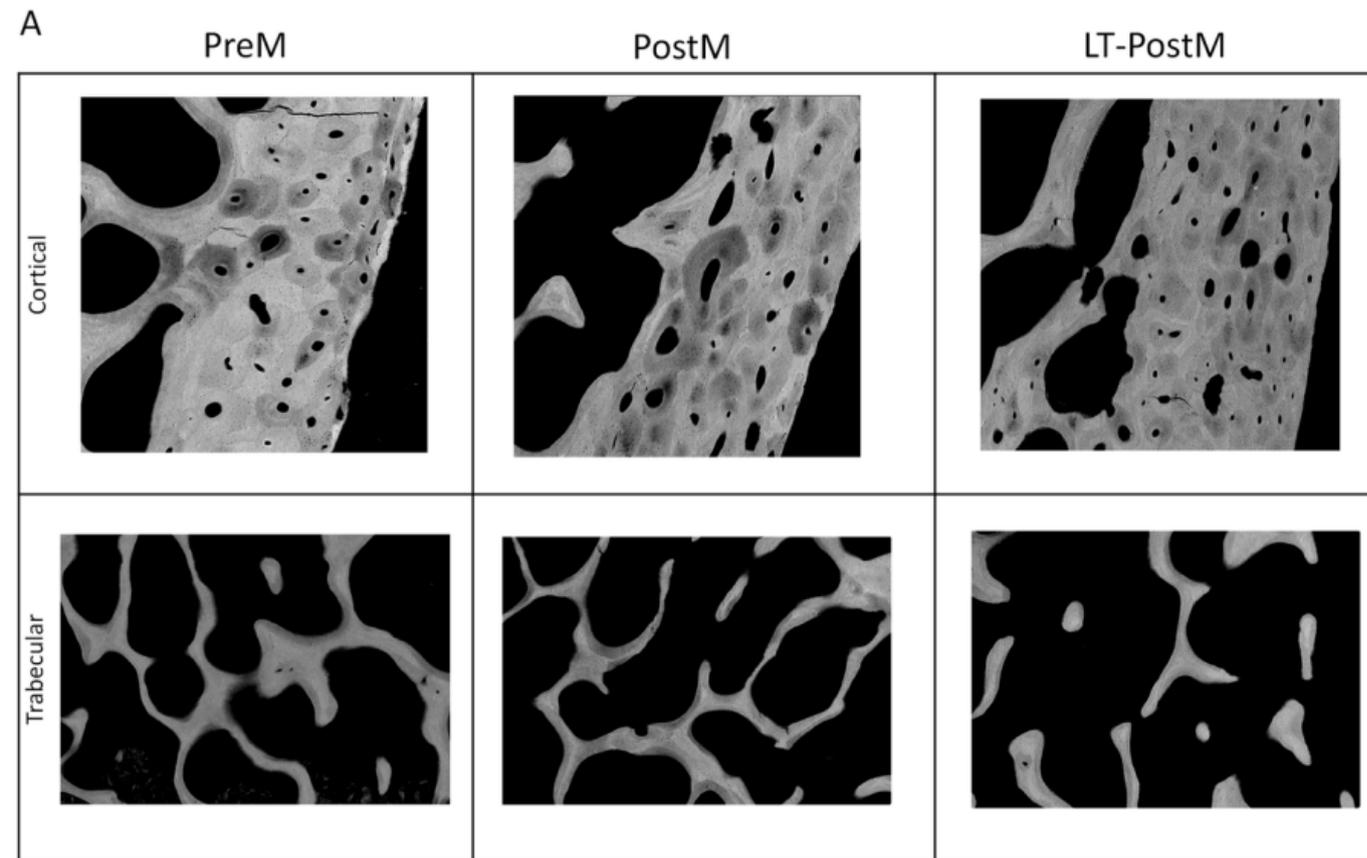
Fig. 1

Fig.2

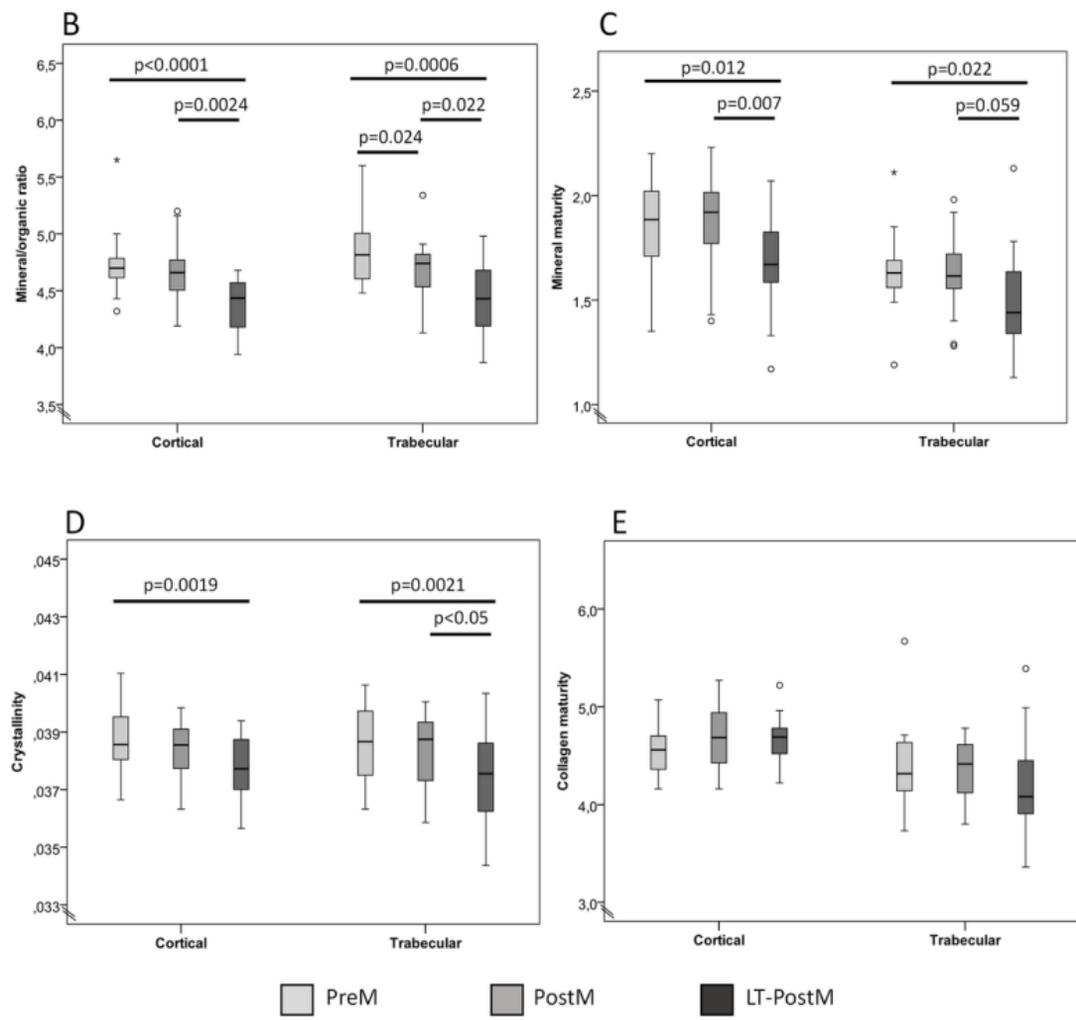
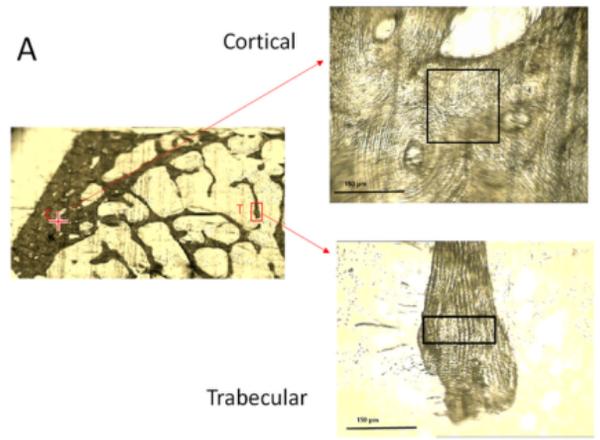


Fig. 3

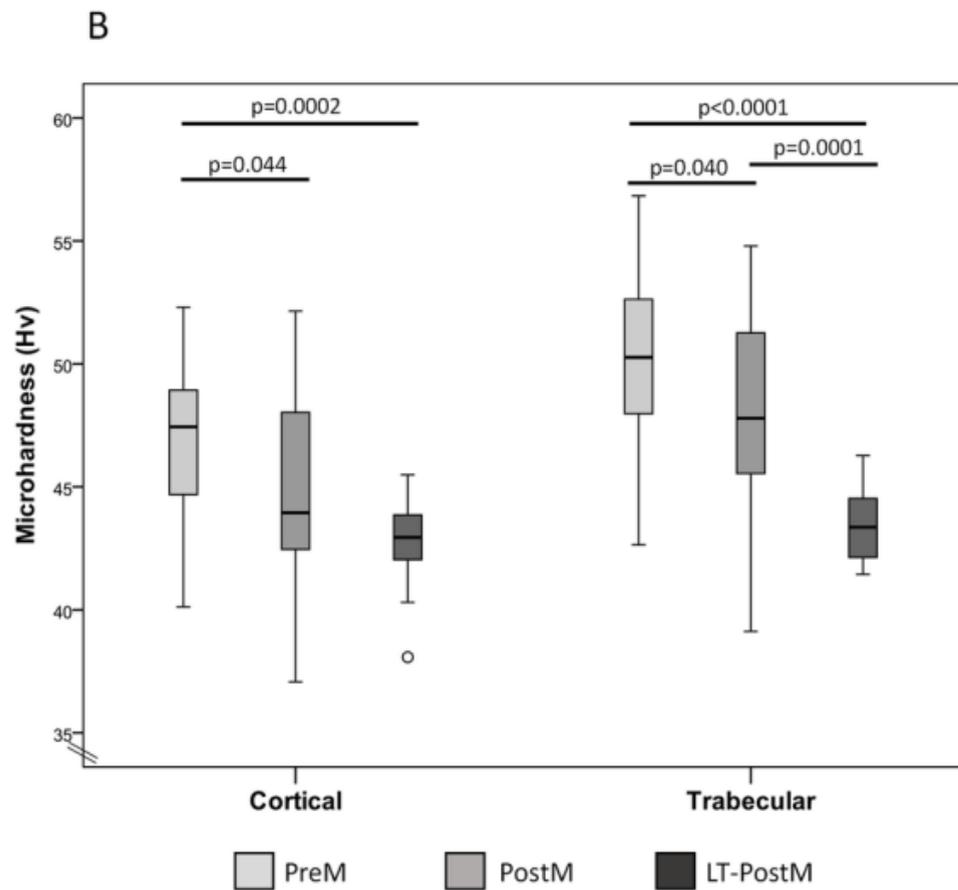
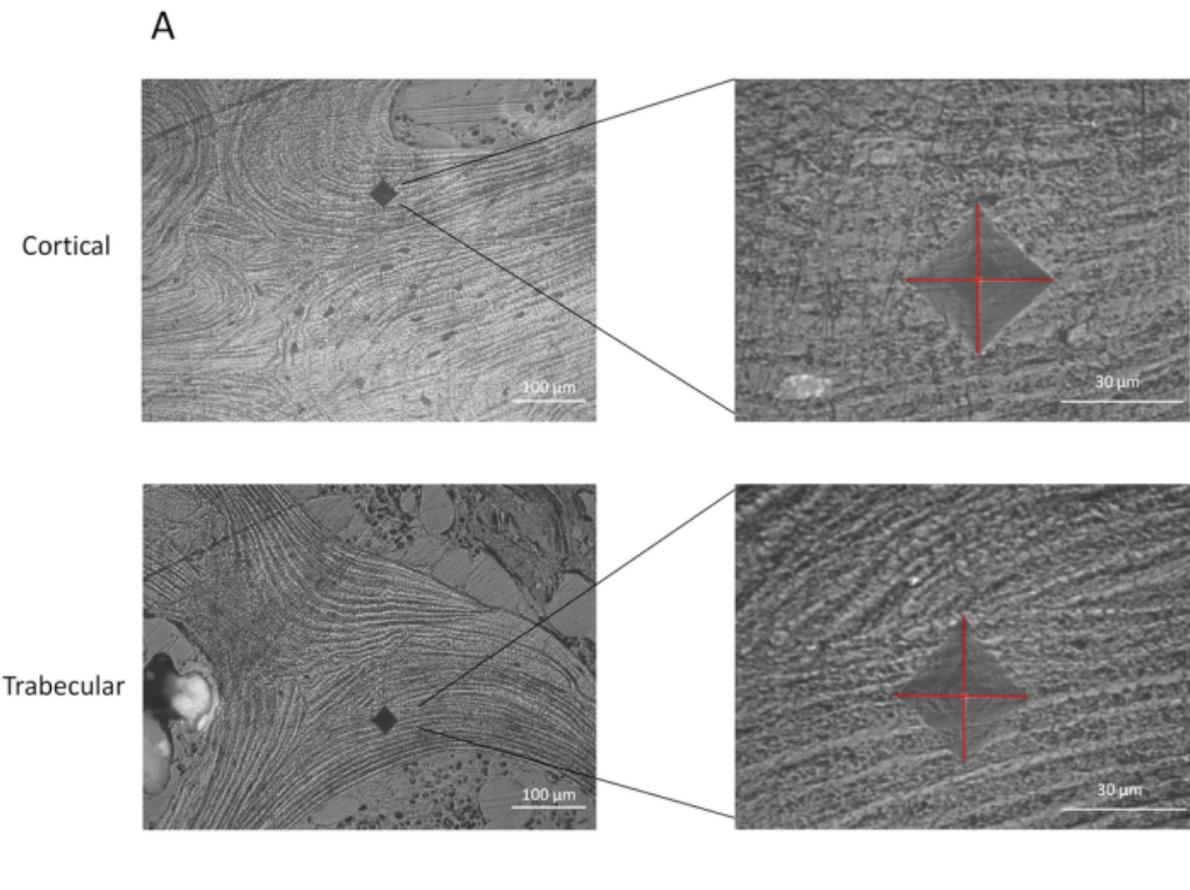


Table 1. Histomorphometric data in healthy premenopausal, post-menopausal and Long-Term post-menopausal women.

Variables	PreMenopausal (n=20) Mean (SD)	PostMenopausal (n=20) Mean (SD)	Difference PreM/PostM Wilcoxon test (<i>p</i> value)	LT-PostMenopausal (n=20) Mean (SD)	Difference PreM/LT-PostM Mann-Whitney test (<i>p</i> value)	Difference PostM/LT-PostM Mann-Whitney test (<i>p</i> value)
Age	49 (2)	55 (2)		60 (7)		
BV/TV (%)	21.9 (6.2)	21.1 (5.2)	NS	22.2 (3.6)	NS	NS
Tb.Th (μm)	155 (41)	148 (38)	NS	143 (31)	NS	NS
Ac.f (#/year)	0.14 (0.11)	0.28 (0.16)	<0.002	0.37 (0.27)	0.019	NS
BFR/BS (mm ³ /mm ² /year)	0.0060 (0.0048)	0.0106 (0.0059)	<0.004	0.0116 (0.0084)	0.0037	NS
BFR/BV (%/year)	0.0745 (0.0552)	0.1376 (0.0587)	0.0019	0.2032 (0.1423)	0.0016	NS
Oc.S/BS (%)	0.089 (0.093)	0.456 (0.394)	0.0003	0.698 (0.703)	0.0001	NS
MS/BS (%)	2.43 (2.14)	3.86 (2.08)	0.0228	4.27 (3.08)	0.0475	NS
W.Th (μm)	42.4 (10)	38.1 (6.9)	NS	31.6 (3.9)	<0.0001	0.0007
FP (year)	2.39 (2.97)	0.89 (0.75)	0.0029	1.00 (0.97)	0.0122	NS

Bone volume/Tissue volume (BV/TV); Trabecular thickness (Tb.Th); Activation Frequency (Ac.f); Bone formation rate/Bone surface (BFR/BS) ; Bone formation rate/Bone volume (BFR/BV); Osteoclast surface/Bone surface (Oc.S/BS), Mineralizing surface/Bone surface (MS/BS); Wall Thickness (W.Th); Formation Period (FP).

Table 2. Summary table illustrating the expected and the observed effects of the influence of a high remodeling activity (assessed by histomorphometry) on trabecular bone matrix quality (60 bone samples).

		Histomorphometry		Area measured in trabecular bone
		Activation Frequency ↑		
		Expected effect	Observed effect	
Bone matrix quality	Degree of Mineralization	↓	↓	Whole bone area
	Heterogeneity Index	↑	↑	Whole bone area
	Microhardness	↓	↓	Randomly selected areas
	Mineral/organic	↓	↓	Randomly selected areas
	Mineral maturity	↓	↔	Randomly selected areas
	Crystallinity	↓	↓	Randomly selected areas
	Collagen maturity	↓	↔	Randomly selected areas