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## Bone matrix quality in paired iliac bone biopsies from postmenopausal women treated for 12 months with strontium ranelate or alendronate

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1 **Bone matrix quality in paired iliac bone biopsies from**  
2 **postmenopausal women treated for 12 months with strontium**  
3 **ranelate or alendronate.**

4  
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24 **1 Highlights**

- 25 • A large number of paired bone biopsies (n=60) were studied  
26 • Alendronate increases mineralization and crystallinity in new bone  
27 • Alendronate increases the relative amount of proteoglycan in new bone  
28 • Strontium ranelate decreases carbonate content and crystallinity  
29 • Strontium promotes bonds with collagen and noncollagenous proteins

30  
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32 Keywords: Mineralization – Collagen – Bone Quality – Alendronate – Strontium – Paired Human Bone  
33 Biopsies

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35  
36

## 37 **2 Abstract**

38 Bone quality is altered mainly by osteoporosis, which is treated with modulators of bone quality.  
39 Knowledge of their mechanisms of action is crucial to understand their effects on bone quality. The goal  
40 of our study was to compare the action of alendronate (ALN) and strontium ranelate (SrRan) on the  
41 determinants of bone quality. The investigation was performed on over 60 paired human iliac biopsies.  
42 Paired samples correspond to biopsies obtained from the same patient, one before treatment (baseline)  
43 and one after 12 months of treatment, in postmenopausal women with osteoporosis. Vibrational  
44 spectroscopy (Raman and FTIRM) and nanoindentation were used to evaluate the effect of both drugs  
45 on bone quality at the ultrastructural level. Outcomes measured by vibrational spectroscopy and  
46 nanoindentation are sensitive to bone age. New bone packets are distinguished from old bone packets.  
47 Thus, the effect of bone age is distinguished from the treatment effect. Both drugs modify the mineral  
48 and organic composition in new and old bone in different fashions after 12 months of administration.  
49 The new bone formed during ALN administration is characterized by an increased mineral content,  
50 carbonation and apatite crystal size/perfection compared to baseline. Post-translational modifications of  
51 collagen are observed through an increase in the hydroxyproline/proline ratio in new bone. The  
52 proteoglycan content is also increased in new bone. SrRan directly modulates bone quality through its  
53 physicochemical actions, independent of an effect on bone remodeling. Strontium cations are captured  
54 by the hydrated layer of the mineral matrix. The mineral matrix formed during SrRan administration has  
55 a lower carbonate content and crystallinity after 12 months than at baseline. Strontium might create  
56 bonds (crosslinks) with collagen and noncollagenous proteins in new and old bone. The nanomechanical  
57 properties of bone were not modified with either ALN or SrRan, probably due to the short duration of  
58 administration. Our results show that ALN and SrRan have differential effects on bone quality in relation  
59 to their mechanism of action.

60

### 61 **3 Introduction**

62 The term “bone quality” describes the composition and architectural properties of bone, which  
63 determine its material and mechanical properties [1]. Bone quality is altered by bone diseases.  
64 Modulators of bone quality are used to treat bone diseases, particularly osteoporosis. Knowledge of  
65 their molecular mechanisms is crucial to obtain an understanding of their effects on bone quality.

66 Bisphosphonates (BPs) have been used for many years as antiresorptive agents for treating bone  
67 diseases, particularly osteoporosis. Among the different BPs available, alendronate (ALN) is the most  
68 commonly used for the treatment of postmenopausal osteoporosis (PMOP) [2]. The mechanism of action  
69 of ALN as an antiresorptive drug acting on osteoclasts is well known [3]. ALN decreases bone resorption  
70 by inhibiting osteoclast formation and function [4]. The effect of ALN on bone quality at the  
71 ultrastructural level has been the subject of several studies involving animal models and humans. In  
72 studies involving human samples and ALN treatment, the duration therapy covered a range of  
73 treatments from 2 to 10 years. For short-term ALN treatment (2-3 years), microradiography, quantitative  
74 backscattered electron imaging (qBEI) and vibrational spectroscopy showed an increase in the mineral  
75 composition and a decrease in the spatial heterogeneity of the bone material properties [5-7]. For long-  
76 term treatment (3-10 years), vibrational spectroscopy and qBEI analysis did not reveal modification of  
77 the mineral and the organic composition at similar bone ages [8-10]. A decrease in the heterogeneity of  
78 the bone material properties was observed in the long-term treatment [11, 12]. Tissue hardness was also  
79 reduced after long-term ALN treatment compared to a placebo [12].

80 Among all available anti-osteoporotic agents, there is a drug that is not considered an anabolic  
81 or anticatabolic agent: strontium ranelate (SrRan). The mechanism of action of  $\text{Sr}^{2+}$  from SrRan on bone is  
82 still being debated [13]. However, studies agree that  $\text{Sr}^{2+}$  is fixed in newly formed bone packets during  
83 treatment. There is no diffusion from newly formed bone packets into old bone packets [14-16]. Three  
84 hypotheses are accepted regarding the location of  $\text{Sr}^{2+}$  in bone mineral. (i) Sr is adsorbed at the surface  
85 of collagen or bone mineral. Sr may have different coordination numbers with surrounding oxygen  
86 atoms; (ii) a slower mechanism of heteroionic substitution occurs, which results in the incorporation of  
87 strontium into the crystal lattice replacing calcium in site (I) or (II); and (iii) Sr is in the hydrated region of  
88 the bone mineral [17, 18]. The effect of SrRan treatment on human bone quality at the microstructural  
89 level was investigated in human samples. The duration therapy covered a range of treatments from 6  
90 months to 8 years. All studies agree about the effect of SrRan on bone properties in human samples. The  
91 maximum amount of Sr fixed in bone reaches less than 1.6% (%wt)Sr/(Sr+Ca) after 3 years of  
92 administration [19, 20]. Vibrational spectroscopy, microradiography, qBEI, small-angle scattering and X-

93 ray diffraction showed no modification of the mineral and collagen cross-links after 3 years of treatment  
94 [14, 15]. Bone mechanical properties, assessed by nanoindentation, showed no difference between  
95 patients treated with SrRan for 3 years versus a placebo [15]. In a comparative study between SrRan and  
96 ALN, histomorphometric analyses showed that bone formation remained higher with a lower diminution  
97 of bone remodeling with SrRan versus ALN [21]. The present study is a follow-up of these  
98 histomorphometric analyses [21].

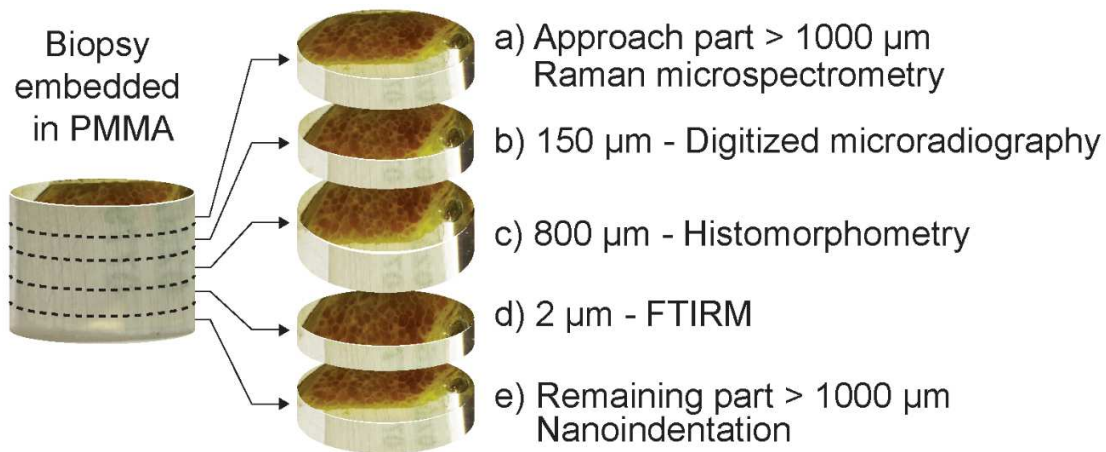
99 Our study aimed to compare bone quality changes induced by two anti-osteoporotic treatments  
100 (ALN and SrRan), which each have their own characteristic mechanism of action. Vibrational  
101 spectroscopy and nanoindentation assessments were performed to evaluate their effects on the mineral  
102 and organic matrix of bone at the ultrastructural level. Vibrational spectroscopy techniques (Raman and  
103 Fourier transform infrared microscopy, FTIRM) were used to study bone at the molecular level [22]. Both  
104 techniques are complementary and describe the molecular composition of mineral and organic  
105 components through physicochemical variables (PPVs). The nanoindentation technique assesses the  
106 tissue hardness, elastic modulus and working energy at the scale of a single BSU. Our study will provide  
107 insights into the differential effect of ALN and SrRan on bone quality in the short term at the  
108 ultrastructural level.

## 109 **4 Materials and methods**

### 110 **4.1 Bone samples**

111 Paired iliac bone biopsies (n=60) were provided by the Institut de Recherches Internationales  
112 Servier (Suresnes, France). These biopsies were obtained from a subset of a double-blind study  
113 comparing the effects of SrRan and ALN on bone histomorphometry [21]. Sixty pairs of bone biopsies (30  
114 pairs SrRan and 30 pairs ALN) were randomly selected among the 387 pairs of bone biopsies from the  
115 initial phase III study [21]. The characteristics of the menopausal women (average age, femoral neck  
116 BMD T-score, total hip BMD T-score, lumbar L1-L4 BMD T-score and time since menopause) are  
117 described in Table 1. Briefly, the 60 osteoporotic women were 50 and 76 years old and were menopausal  
118 for at least 3 years. The ALN group had an average age of 64.4 years, and the SrRan group had an  
119 average age of 63.3 years. No statistically significant difference was found between the ALN and SrRan  
120 groups regarding the main characteristics of the population. A first transiliac bone biopsy was performed  
121 with a 7.5-mm inner diameter trephine in all patients at baseline. A second bone biopsy specimen was  
122 collected from the opposite side after 12 months of administration. The dosing regimen was set at 2 g  
123 per day for SrRan and 70 mg once a week for ALN. The 60 paired biopsies were divided into 2 groups

124 according to the treatment administered: ALN and SrRan. Each group included 30 paired samples. A  
125 paired sample corresponded to a biopsy obtained from the same patient at baseline (M0) and a biopsy  
126 obtained after 12 months of treatment (M12). After fixation with 70% ethanol and dehydration in 100%  
127 ethanol, biopsies were embedded in polymethylmethacrylate (PMMA). Each block of PMMA was cut into  
128 5 parts with specific thicknesses adapted to the technique of analysis. Three of 5 parts were used in the  
129 present study: the approach part, a 2  $\mu\text{m}$ -thick part and the remaining part (Figure 1 a, d and e). The 3  
130 parts were not stained.



131  
132 **Figure 1:** Each embedded biopsy was cut into 5 parts with a specific thickness adapted to the technique of analysis. a) For the  
133 “approach part”, the thickness was greater than 1000  $\mu\text{m}$  and dedicated to Raman microspectrometry. b) The thickness was ~  
134 150  $\mu\text{m}$  and dedicated to digitized microradiography. The results of this technique are not included in the actual article. c) The  
135 thickness was ~ 800  $\mu\text{m}$  and dedicated to histomorphometry. The results are presented in the article by *Chavassieux et al*  
136 [21]. d) The thickness was ~ 2  $\mu\text{m}$  and dedicated to FTIRM analysis. e) The thickness was greater than 1000  $\mu\text{m}$  and dedicated  
137 to nanoindentation analysis.  
138

139 The samples were divided into 2 sets. The first set was used for vibrational spectroscopic analysis  
140 and included 30 paired samples per treatment. For the FTIRM analysis, undecalcified parts (2  $\mu\text{m}$  thick)  
141 were cut from the first set with a Polycut E microtome (Reichert-Jung, Leica, Germany) and stored  
142 between 2 slides. Raman microspectroscopy was performed on the “approach part”. The surface was  
143 polished using abrasive paper with decreasing grains (30, 12, 3 and 0.3  $\mu\text{m}$ ). The second set was  
144 composed of the remaining parts and analyzed by nanoindentation. During the time frame of this study,  
145 only 19 paired bone biopsies per treatment were analyzed because nanoindentation was time  
146 consuming. Sections of biopsies were polished, finished with a 0.25 mm diamond solution, and  
147 rehydrated in a saline solution for 12 hours at 22°C using a standard protocol. The design of the study is  
148 presented in Figure 2.

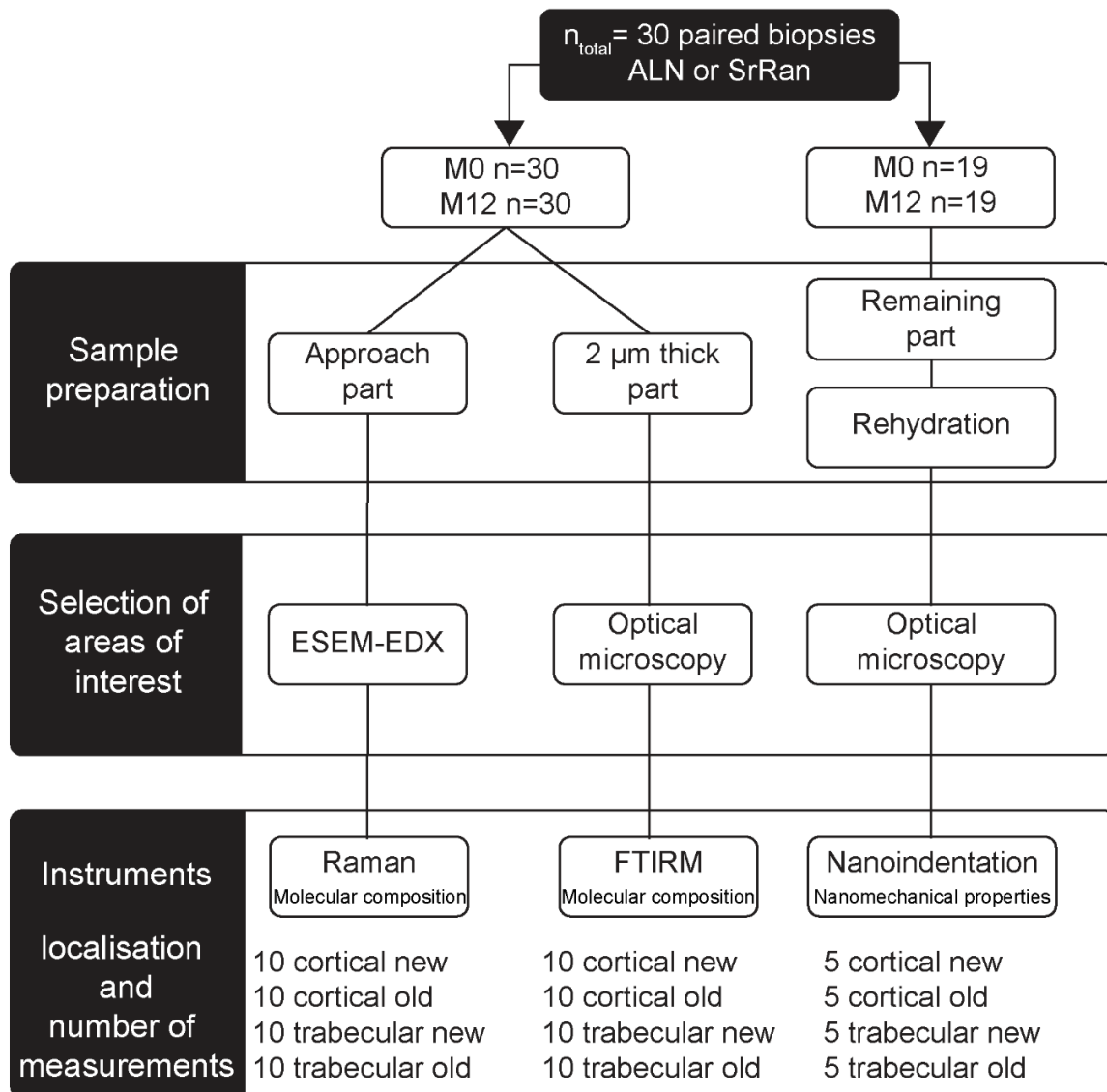
149

150 **Table 1: Averaged characteristics of the patients at baseline. The comparison of the characteristics of both groups was**  
 151 **performed with a t-test. There was no difference between the groups according to age, femoral neck BMD T-score, total hip**  
 152 **BMD T-score, lumbar L1-L4 BMD T-score or the time since menopause.**

	ALN	SrRan	p-value
Age (years)	64.4 (±6.35)	63.3 (±7.77)	0.57
Femoral neck BMD T-score	-2.14 (±0.71)	-2.07 (±0.63)	0.71
Total hip BMD T-score	-1.73 (±0.86)	-1.49 (±0.75)	0.25
Lumbar L1-L4 BMD T-score	-2.96 (±0.81)	-2.74 (±0.65)	0.26
Time since menopause (years)	16.24 (±6.73)	13.84 (±7.61)	0.20
Demographic origin (n [%])			
North America	14 [23]	12 [20]	
South America	1 [2]	2 [3]	
Europe	15 [25]	16 [27]	

153 **BMD= Bone Mineral Density.**

154 **Values are expressed as means with standard deviation (±SD) or percentage (n[%]) of the total number of biopsies (n=60).**



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 Figure 2: Description of the design of the study for each treatment (ALN or SrRan). Each treatment was composed of 30 paired biopsies embedded in PMMA. A paired biopsy corresponds to a biopsy at baseline (before the treatment, M0) and a biopsy after 12 months (M12) of administration of ALN or SrRan. Group M0 was composed of 30 biopsies, and group M12 was composed of 30 biopsies. Each biopsy was cut into 5 parts (Figure 1), and 3 parts were analyzed in this work. The approach parts were imaged by environmental scanning electron microscopy coupled with energy dispersive X-ray (ESEM-EDX) to distinguish new from old bone packets. All paired approach parts (n=30) were analyzed by Raman microspectroscopy. The 2 μm-thick parts were observed by optical microscopy to distinguish new from old bone packets. All paired 2 μm-thick parts (n=30) were analyzed by FTIRM. The remaining paired parts were observed by optical microscopy to distinguish new from old bone packets. Nineteen of the 30 paired remaining parts were rehydrated and analyzed by nanoindentation.

## 165 4.2 Raman spectroscopy and physicochemical variables

166 The Raman spectrometer was a LabRAM HR800 (HORIBA, Jobin-Yvon, France). The acquisition  
 167 time per spectrum was the average of 4 acquisitions of 30 seconds each (120 sec in total). The laser  
 168 wavelength was 785 nm and the laser power output was 100 mW. The DuoScan mode was set to scan a



169 surface of  $30 \times 30 \mu\text{m}^2$  to ensure a similar spatial resolution as the FTIRM analysis [23]. One Raman  
170 spectrum corresponds to one bone packet. Five spectra were acquired on 5 different new bone packets  
171 from each cortical region (left and right). Ten spectra were acquired from 10 different new trabecular  
172 bone packets. A total of 20 spectra (10 cortical and 10 trabecular) were acquired in new bone packets for  
173 each sample. The same acquisition protocol was repeated on the old bone packets from each biopsy,  
174 resulting in 20 measurements of old bone (10 cortical and 10 trabecular). The localization procedure is  
175 detailed in section 4.5 (Figure 3). After the acquisition, a Savitzky–Golay smoothing filter (filter width: 3;  
176 and polynomial order: 2) was applied to all Raman spectra. The intensities and areas were integrated  
177 over defined Raman shift regions in the spectrum using a sum filter. The filter calculates the intensities  
178 and areas within the chosen borders, and the background is subtracted by taking a local piecewise linear  
179 baseline from the first to the second border [24]. Six physicochemical variables (PPVs) were calculated  
180 for each spectrum [25, 26]: the mineral/organic ratio = ratio of area of  $\nu_1\text{PO}_4$  ( $960 \text{ cm}^{-1}$ , peak-ROI 900-  
181  $990 \text{ cm}^{-1}$ ) to the area of  $\delta(\text{CH}_2)$  ( $1450 \text{ cm}^{-1}$ , peak-ROI 1434 -1490  $\text{cm}^{-1}$ ); carbonation type-B = ratio of area  
182 of type-B  $\text{CO}_3^{2-}$  ( $1070 \text{ cm}^{-1}$ , peak-ROI 1052–1092  $\text{cm}^{-1}$ ) and area of  $\nu_1\text{PO}_4$ ; crystallinity, the full width at  
183 half maximum intensity (FWHM) of  $\nu_1\text{PO}_4$ ; the hydroxyproline/proline ratio = ratio of intensity of  
184 hydroxyproline ( $875 \text{ cm}^{-1}$ , peak-ROI 828-898  $\text{cm}^{-1}$ ) and intensity of proline ( $855 \text{ cm}^{-1}$ , peak-ROI 828-898  
185  $\text{cm}^{-1}$ ); collagen maturity = ratio of the intensity of  $1660 \text{ cm}^{-1}$  (peak-ROI 1615 -1705  $\text{cm}^{-1}$ ) and intensity of  
186  $1690 \text{ cm}^{-1}$  (peak-ROI 1615 -1705  $\text{cm}^{-1}$ ) [27]; and the relative proteoglycan (PG) content = ratio of areas of  
187 PG (peak-ROI 1365–1390  $\text{cm}^{-1}$ ) to the area of amide III band (peak-ROI 1243–1269  $\text{cm}^{-1}$ ) [28]. An example  
188 of a raw Raman spectrum with bands assignments and local piecewise linear baseline is shown in the  
189 supplementary file (Figure S1-a).

### 190 **4.3 FTIRM and physicochemical variables**

191 FTIRM analysis was performed in transmission mode on  $2 \mu\text{m}$ -thick sections with a Spectrum 100  
192 spectrometer coupled with an AutoImage GXII microscope (PerkinElmer GXII Auto-image Microscope)  
193 equipped with a wideband detector (mercury–cadmium–telluride) ( $7800\text{--}400 \text{ cm}^{-1}$ ). Each spectrum was  
194 collected at a  $45 \times 35 \mu\text{m}^2$  spatial resolution with a  $4 \text{ cm}^{-1}$  spectral resolution and an average of 50 scans.  
195 The contributions of air and PMMA (by nullifying the main peak of PMMA at  $1730 \text{ cm}^{-1}$ ) were subtracted  
196 from each raw spectrum, and the baseline (quadratic function) was corrected. An example of a raw and  
197 corrected spectra with the assignment of bands is shown in the supplementary file (Figure S1-b). Each  
198 measurement was performed on both new cortical and trabecular bone samples. Similar to Raman  
199 analysis, one FTIRM spectrum corresponds to one bone packet. Five acquisitions were performed on 5  
200 different new bone packets from each cortical region (left and right). Ten spectra were acquired from 10

201 different new trabecular bone packets. A total of 20 spectra (10 cortical and 10 trabecular) were  
202 acquired in new bone packets for each sample. The same acquisition protocol was repeated on the old  
203 bone packets from each biopsy, resulting in 20 measurements of old bone (10 cortical and 10  
204 trabecular). The localization procedure is detailed in section 4.5 (Figure 4-a). Finally, each spectrum was  
205 deconvoluted by using the peak fitting method with GRAMS/AI software (Thermo Galactic, Salem, NH,  
206 USA). Regarding the  $\nu_1\nu_3\text{PO}_4$  vibration, five subbands were used (1110, 1082, 1060, 1030, 962  $\text{cm}^{-1}$ ), and,  
207 for the  $\nu_4\text{PO}_4$  vibration, four subbands were used (604, 577, 563, 552  $\text{cm}^{-1}$ ). The amide I vibration was  
208 curve-fitted into 3 main components, 1690  $\text{cm}^{-1}$ , 1660  $\text{cm}^{-1}$  and 1633  $\text{cm}^{-1}$ , and 6 other peaks  
209 corresponding to the other amide vibrations were added. For the amide vibration, a total of 9 peaks  
210 were fitted altogether [29-32]. Five variables were calculated per spectrum: mineral/organic ratio = ratio  
211 of the integrated area under the curve for  $\nu_1\nu_3\text{PO}_4$  (1184-910  $\text{cm}^{-1}$ ) and the integrated area under the  
212 curve for amide I (1730-1592  $\text{cm}^{-1}$ ); mineral maturity = ratio of the area under the curve for  $\nu_1\nu_3\text{PO}_4$  at  
213 1030  $\text{cm}^{-1}$  (apatitic phosphates) and the area under the curve for  $\nu_1\nu_3\text{PO}_4$  at 1110  $\text{cm}^{-1}$  (nonapatitic  
214 phosphates); crystallinity = inverse of the width at the half-height of the peak at 604  $\text{cm}^{-1}$  ( $\nu_4\text{PO}_4$ ); and  
215 carbonation= ratio of the integrated area under the curves for  $\nu_2\text{CO}_3$  (862-894  $\text{cm}^{-1}$ ) and  $\nu_1\nu_3\text{PO}_4$  (1184-  
216 910  $\text{cm}^{-1}$ ). FTIRM assesses the total carbonate contents (types A + B + labile), while Raman spectroscopy  
217 assesses the type-B carbonate contents. Collagen maturity is defined as the ratio of the area at 1660  $\text{cm}^{-1}$   
218 (amide I) to the area at 1690  $\text{cm}^{-1}$  (amide I).

#### 219 **4.4 Nanomechanical properties: nanoindentation analysis**

220 Nanoindentation tests were used to evaluate the material-level properties of bone tissue. The elastic  
221 modulus, tissue hardness, working energy and elastic energy of the bone were determined for  
222 rehydrated bone tissue samples. A nanoindentation tester (NHT, CSM Instruments, Peseux, Switzerland)  
223 was used as follows: force-displacement data of a pyramidal diamond indenter that was pressed onto a  
224 material were recorded as previously described [33]. An original Berkovich tip with a three-sided  
225 pyramid characterized by a half angle of 65.3 degrees was used. The indenter tip was loaded at a given  
226 depth into the sample, and the load was then held constant, leading to creeping of the material below  
227 the tip. This results in a combination of elastic and postyield deformation, which is analyzed to obtain the  
228 elastic modulus and tissue hardness as well as the working energy. Tissue hardness is interpreted as the  
229 mean pressure that the material can resist and is calculated as the ratio of the maximum force to the  
230 contact area. The elastic modulus is defined by the initial slope of the unloading section of the curve.  
231 Finally, the working energy is calculated as the surface between the load-displacement curves, as well as  
232 the plastic and elastic energy. The details of the variables are presented in the supplementary files. The

233 sections of the human biopsies were polished and finished with a 0.25 mm diamond solution, and the  
234 samples were rehydrated following a standardized protocol in saline solution for 12 hours at 22 °C. The  
235 mechanical tests were conducted at locations comparable to those analyzed using Raman spectroscopy  
236 and FTIRM. One indent corresponds to one bone packet. Five indents were generated in 5 different  
237 cortical new bone packets (over left and right cortices). Five indents were generated in 5 different  
238 trabecular new bone packets. It represents a total of 10 indents in the new bone (5 cortical and 5  
239 trabecular). The same procedure was performed in cortical and trabecular old bone. A total of 10 indents  
240 were obtained in old bone (5 cortical and 5 trabecular). The localization procedure is detailed in section  
241 4.5 (Figure 4-d). The indents were set to a 900 nm depth with an approximate speed of 76 mN/min for  
242 both loading and unloading, and, at maximum load, a 10 second holding period was applied. Finally, the  
243 limit of the maximal allowable thermal drift was set to 0.1 nm/s.

#### 244 **4.5 Localization of areas with comparable bone age**

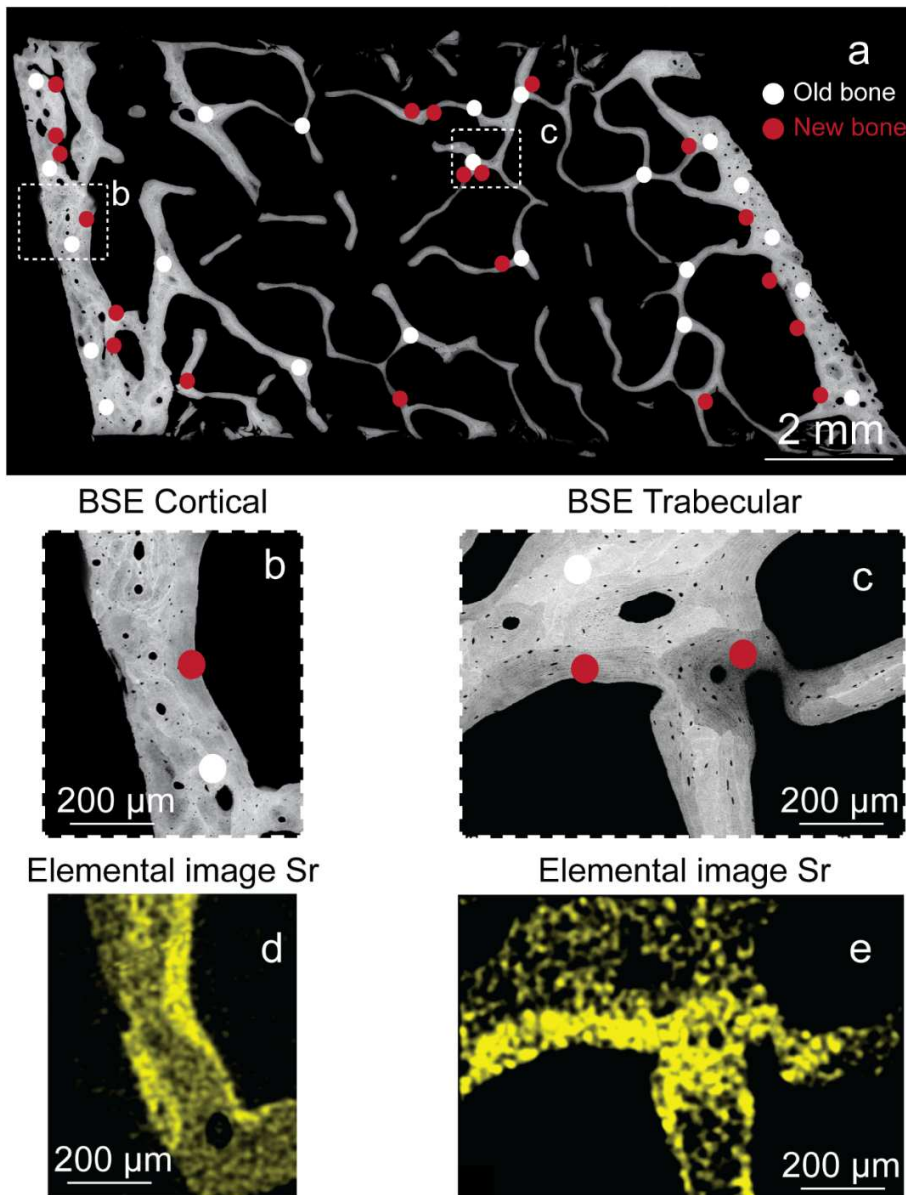
245 Outcomes measured by vibrational spectroscopy (Raman and FTIRM) and nanoindentation are  
246 sensitive to bone age. Bone packets must be distinguished to compare bone areas with similar bone  
247 ages. Therefore, the effect of bone age is dissociated from the effect of the treatment. Initially, all  
248 patients received double tetracycline labeling before bone biopsies [21]. Unfortunately, the tetracycline  
249 labels were not clearly visible. Two procedures were performed to locate the bone areas depending on  
250 the bone age. Each procedure was adapted according to the sample preparation constraints.

251 The first procedure was based on an environmental scanning electron microscope coupled with  
252 an energy dispersive spectroscopy detector (ESEM-EDX). Prior to the acquisition of the Raman spectra,  
253 the biopsies were analyzed using ESEM-EDX. Back scattered electron images (BSE) were used to  
254 distinguish bone packets with comparable mineralization levels within each sample and thus comparable  
255 bone ages. Figure 3 illustrates an example of selected locations. Areas with a dark gray level were  
256 distinguished from areas with a light gray level in the ALN M0, ALN M12, and SrRan M0 samples. This  
257 distinction allowed us to identify recently formed bone packets (Figure 3-dark gray-red circles) and old  
258 interstitial bone packets (Figure 3-light gray-white circles) [34]. Elemental images of Sr<sup>2+</sup> were acquired  
259 from SrRan M12 samples to locate bone packets in which Sr was fixed (Figure 3-d and e). The BSE images  
260 and the elemental images of Sr<sup>2+</sup> were used as a guide for Raman analysis. Throughout the manuscript,  
261 the term “new bone” refers to recently formed bone packets, and the term “old bone” refers to old  
262 interstitial bone.

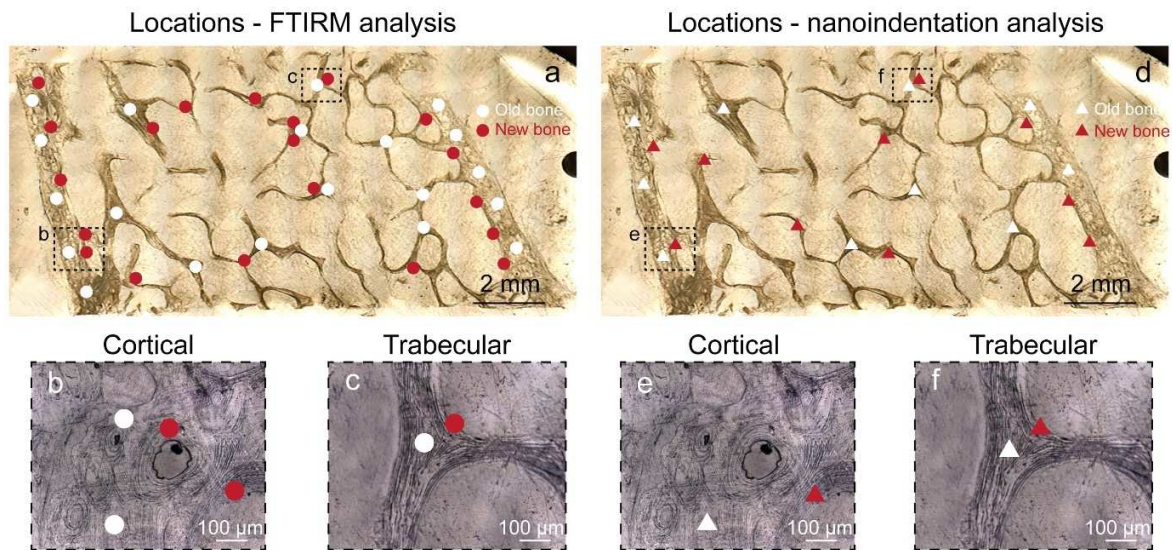
263 The second procedure was based on optical microscopy. Prior to FTIRM and nanoindentation  
264 analyses, optical microscopy was used to localize bone packets with similar bone ages. Bone packets with

265 similar bone ages have characteristic anatomical locations. The location of the bone packets was  
266 determined according to the following procedure and was used as a guide for FTIRM and  
267 nanoindentation analyses. Figure 4 illustrates an example of selected locations. In cortical bone, recently  
268 formed bone packets were located in the endosteal area (red circles and diamonds). Old interstitial bone  
269 packets were located in the middle of the cortical bone (white circles and diamonds). In trabecular bone,  
270 recently formed bone packets were located in the periphery (red circles and diamonds). Old interstitial  
271 bone packets were located in the middle of the trabeculae (white circles and diamonds).

### Locations - Raman analysis



272  
 273 **Figure 3: Example of localization of new and old bone packets analyzed by Raman spectroscopy.** a) BSE image of an entire  
 274 biopsy of the SrRan group. Circles in red and white represent new and old bone packets, respectively. Bone packets with dark  
 275 gray correspond to a low level of mineralization (new bone), and bone packets with light gray correspond to a high level of  
 276 mineralization (old bone). b and d) High magnification of a bone packet and the corresponding elemental image Sr in the  
 277 cortical bone. c and e) High magnification of a bone packet and the corresponding elemental image Sr in the trabecular bone.  
 278 In elemental image Sr, the area in strong yellow corresponds to the presence of Sr in new bone, and the area in light yellow  
 279 corresponds to the noise. The BSE images and the elemental images Sr were used to guide Raman analysis.  
 280



281  
 282 **Figure 4:** Example of the localization of new and old bone packets analyzed by FTIRM and nanoindentation. a) and d) optical  
 283 image of an entire SrRan biopsy. Circles and diamonds represent examples of locations analyzed by FTIRM and  
 284 nanoindentation, respectively. Magnification of a bone packet in b) cortical and c) trabecular bone for FTIRM analysis.  
 285 Magnification of a bone packet in e) cortical and f) trabecular bone for nanoindentation analysis. The circles and diamonds in  
 286 red represent locations of new bone. The circles and diamonds in white represent locations of old bone. Optical microscopy  
 287 was used to guide FTIRM and nanoindentation analysis.  
 288

#### 289 4.6 Bone sample quality analysis

290 The embedded biopsies were stored in the dark at ambient temperature [21]. Bone biopsies have been  
 291 embedded for more than 5 years. Five elements were checked to evaluate whether long-term storage  
 292 had an influence on the outcomes measured. First, the Raman and FTIRM spectra were in agreement  
 293 with the reference spectra of bone. Second, the values of all PPVs were in agreement with PPVs from  
 294 similar studies in the literature. Third, we also checked the coherence of the data when comparing PPVs  
 295 according to the bone age (new bone versus old bone). The comparison of the well documented PPVs of  
 296 new bone versus old bone gave results in agreement with the literature. PPVs such as the mineral/matrix  
 297 ratio, carbonation and crystallinity were lower in new bone than in old bone. Fourth, EDX analysis  
 298 confirmed the presence of  $Sr^{2+}$  in newly formed bone tissue after administration of SrRan. Fifth, the  
 299 tissue hardness of bone is independent of the duration of embedding (article submitted).

#### 300 4.7 Statistical analysis

301 The change in each physicochemical variable after 12 months were compared between the two  
 302 treatments and the two locations using a linear mixed model (covariance pattern model) with treatment,  
 303 location, treatment \* location interaction and baseline value as fixed effects. The patients were

304 considered random effects. In the case of a significant interaction term, a post hoc comparison between  
305 treatments was performed. In the case of a nonsignificant interaction term, the same model was  
306 performed without the treatment \* location interaction to estimate the treatment effect and the  
307 location effect.

308 Data were analyzed using SAS software (SAS Institute Inc., Cary, NC, USA), and all statistical tests were  
309 performed with a 2-tailed alpha risk of 0.05.

## 310 **5 Results**

311 The physicochemical variables (PPVs) were evaluated on new bone and old bone separately. The  
312 changes in the PPVs between baseline and after 12 months were compared according to the ALN or  
313 SrRan group. The Table 2 shows the averaged changes in PPVs in new bone independent of the location  
314 (cortical and trabecular). Statistical analysis revealed that only 2 of 15 PPVs were dependent on the  
315 location in new bone. Table 3 shows the averaged changes of both PPVs in new bone as a function of the  
316 location. Table 4 shows the averaged changes in the PPVs in old bone independent of the location. No  
317 PPVs were found to be dependent on the location in old bone. The effects of ALN and SrRan bone quality  
318 were evaluated by comparing the PPVs according to the bone age. Thus, the results and the discussion  
319 sections are presented according to the bone age (new bone and old bone).

### 320 **5.1 Comparison of the results obtained in the new bone**

321 The comparisons between the two treatments on the new bone are presented in Table 2.

322 **The mineral composition is modified after 12 months of treatment with ALN or SrRan.** The evolution of  
323 all of the mineral PPVs in the new bone was different according to the treatment (all  $p < 0.001$ ) except for  
324 mineral maturity ( $p = 0.62$ ). The mineral/organic ratio (Raman) was increased by ALN but decreased  
325 during SrRan administration. The mineral/organic ratio (FTIRM) was increased by ALN but not modified  
326 by SrRan. Carbonation type B and carbonation (FTIRM) were not modified by ALN but were decreased by  
327 SrRan. The crystallinity (Raman and FTIRM) was increased by ALN and decreased by SrRan. The mineral  
328 maturity was not significantly modified regardless of the treatment.

329 **The organic composition was not deeply modified by either treatment after 12 months in new bone.**

330 Two organic PPVs were significantly different between the two treatments: the hydroxyproline/proline  
331 ratio ( $p = 0.041$ ) and PG/AmideIII ( $p < 0.001$ ). The hydroxyproline/proline ratio was significantly increased  
332 by ALN and not modified by SrRan. PG/AmideIII was increased by ALN and decreased by SrRan in new  
333 bone within the same range. Collagen maturity (Raman and FTIRM) was not modified by either  
334 treatment in new bone after 12 months (Table 2).

335 **The nanomechanical properties were not modified in new bone.** The change in the nanomechanical  
336 PPV was not significantly different between the two treatments. The nanomechanical properties were  
337 not significantly modified in new bone by ALN or by SrRan treatment.

338 **Interactions between treatment and location.** Significant interactions were found between treatment  
339 and location (trabecular/cortical) in new bone for carbonation type B (Raman) ( $p=0.040$ ) and mineral  
340 maturity (FTIRM) ( $p=0.012$ ) only (Table 3). This result indicates that the effect of the treatment is a  
341 function of the location for both variables. A significant increase in carbonation type B was observed in  
342 cortical bone after ALN treatment but not in trabecular bone. In SrRan treatment, a significant decrease  
343 in carbonation type B was observed for cortical and trabecular bone. Mineral maturity was not modified  
344 in the cortical bone with ALN treatment but increased significantly in the cortical bone with SrRan  
345 treatment. Conversely, it decreased in the trabecular bone significantly with SrRan treatment but  
346 nonsignificantly with ALN treatment.

## 347 **5.2 Comparison of the results obtained in the old bone**

348 The comparisons between the two treatments in the old bone are presented in Table 4.

349 **The mineral composition was modified in old bone by ALN but not by SrRan.** The mineral/organic ratio  
350 (Raman) and crystallinity (Raman) were significantly increased in ALN old bone. The mineral PPVs were  
351 not modified by SrRan after 12 months of administration. No significant difference was found between  
352 the two treatments for the change in mineral PPVs in old bone.

353 **The organic composition was modified in old bone by ALN and SrRan.** In old bone, a significant  
354 difference was found between the 2 treatments for 2 variables: hydroxyproline/proline ratio ( $p<0.001$ )  
355 and PG/AmideIII ( $p<0.001$ ). The hydroxyproline/proline ratio was significantly increased by both  
356 treatments, and the increase was lower with ALN than with SrRan. PG/AmideIII was not modified by ALN  
357 but was significantly decreased by SrRan administration. This variable was decreased within the same  
358 range in SrRan old bone as in SrRan new bone. The change in collagen maturity (Raman and FTIRM) was  
359 not significantly different between the two treatments, and no significant change was observed for  
360 either treatment.

361 **The nanomechanical properties were not modified in old bone.** No significant difference was found  
362 between the two treatments for the change in nanomechanical PPV in old bone.

363 **Interactions between treatment and location.** No significant interaction was found between treatment  
364 and location in old bone.

365



366 Table 2: Comparison of the changes in variables ( $\Delta$ PPV) from baseline to 12 months between the two treatments in new  
 367 bone. The mean  $\Delta$ PPV and the statistical evaluation were calculated independently of the location of the bone (cortical and  
 368 trabecular).  
 369

	mean $\Delta$ PPVs - new bone	ALN	SrRan	p-value
		Mean $\Delta$ PPV (95% CI)	Mean $\Delta$ PPV (95% CI)	
MINERAL	Mineral/Organic (Raman)	<b>+0.49 (+0.34 to +0.64)<sup>a</sup></b>	<b>-0.27 (-0.42 to -0.13)<sup>b</sup></b>	<b>&lt;0.001</b>
	Mineral/Organic (FTIRM)	<b>+0.34 (+0.25 to +0.42)<sup>b</sup></b>	+0.01 (-0.07 to +0.10)	<b>&lt;0.001</b>
	Carbonation-type-B $\times 10^{-3}$ (Raman)	+1.67 (-0.21 to +3.54)	<b>-10.72 (-12.59 to -8.86)<sup>a</sup></b>	<b>&lt;0.001</b>
	Carbonation $\times 10^{-3}$ (FTIRM)	+0.25 (-0.08 to +0.57)	<b>-0.64 (-0.96 to -0.31)<sup>a</sup></b>	<b>&lt;0.001</b>
	Crystallinity $\times 10^{-3}$ (Raman)	<b>+0.82 (+0.61 to +1.01)<sup>a</sup></b>	<b>-1.14 (-1.34 to -0.95)<sup>a</sup></b>	<b>&lt;0.001</b>
	Crystallinity $\times 10^{-3}$ (FTIRM)	<b>+0.86 (+0.44 to +1.29)<sup>a</sup></b>	<b>-0.45 (-0.87 to -0.02)<sup>c</sup></b>	<b>&lt;0.001</b>
	Mineral maturity $\times 10^{-3}$ (FTIRM)	-9.53 (-86.15 to +67.08)	+17.43 (-59.18 to +94.05)	0.62
	ORGANIC	HydroxyPro/Pro. $\times 10^{-3}$ (Raman)	<b>+8.66 (+2.13 to +15.18)<sup>c</sup></b>	-1.06 (-7.56 to +5.43)
Collagen maturity $\times 10^{-3}$ (Raman)		-11.06 (-25.11 to +2.99)	-7.25 (-21.23 to +6.73)	0.71
Collagen maturity $\times 10^{-3}$ (FTIRM)		+60.01 (-98.02 to +218.00)	+90.65 (-67.39 to +248.70)	0.78
PG/Amide III $\times 10^{-3}$ (Raman)		<b>+10.08 (+5.60 to +14.56)<sup>a</sup></b>	<b>-9.33 (-13.80 to -4.87)<sup>a</sup></b>	<b>&lt;0.001</b>
NANO.	Elastic modulus (GPa)	+0.44 (-0.16 to +1.04)	+0.41 (-0.16 to +0.97)	0.93
	Tissue hardness (mPa)	+13.22 (-6.72 to +33.17)	+21.73 (+2.89 to +40.58)	0.53
	Working energy (pJ)	-83.24 (-214.45 to +47.97)	+17.28 (-106.26 to +140.84)	0.26
	Elastic energy (pJ)	-6.76 (-42.74 to +29.21)	+17.61 (-16.29 to +51.53)	0.32

370  
 371 The  $\Delta$ PPV corresponds to the difference in the PPV between M0 and M12 adjusted on the PPV value at M0. The  
 372 p-value corresponds to the comparison of the  $\Delta$ PPV between the two treatments.

373 95% CI: 95% confidence interval

374 The labels a:  $p < 0.001$ ; b:  $p < 0.01$ ; c:  $p < 0.05$  correspond to the comparison of the  $\Delta$ PPV with zero.

375  
 376  
 377

378 Table 3: Comparison of the changes in variables ( $\Delta$ PPV) from baseline to 12 months between the two treatments according  
 379 to a cortical or trabecular location in new bone. The effects of ALN and SrRan are functions of the location (cortical and  
 380 trabecular) only for carbonation type B and mineral maturity. The other PPVs do not depend on the location.  
 381

	mean $\Delta$ PPVs - new bone	Location	ALN	SrRan	p-value
			Mean $\Delta$ PPV (95% CI)	Mean $\Delta$ PPV (95% CI)	
MINERAL	Carbonation-type-B $\times 10^{-3}$ (Raman)	Cortical	<b>+2.30 (+0.11 to +4.50)<sup>c</sup></b>	<b>-11.86 (-14.08 to -9.64)<sup>a</sup></b>	<b>0.040</b>
		Trabecular	+1.12 (-1.03 to +3.27)	<b>-9.73 (-11.84 to -7.61)<sup>a</sup></b>	<b>0.040</b>
	Mineral maturity $\times 10^{-3}$ (FTIRM)	Cortical	+24.02 (-67.67 to +115.70)	<b>+143.6 (+51.13 to +236.0)<sup>b</sup></b>	<b>0.012</b>
		Trabecular	-65.51 (-147.0 to +16.03)	<b>-86.30 (-167.1 to -5.49)<sup>c</sup></b>	<b>0.012</b>

382  $\Delta$ PPV corresponds to the difference in the PPV between M0 and M12 adjusted on the PPV value at M0. The p-value  
 383 corresponds to the comparison of the  $\Delta$ PPV between the two treatments.

384 95% CI: 95% confidence interval

385 The labels a:  $p < 0.001$ ; b:  $p < 0.01$ ; c:  $p < 0.05$  correspond to the comparison of the  $\Delta$ PPV with zero.

386 Table 4: Comparison of the changes in variables ( $\Delta$ PPV) from baseline to 12 months between the two treatments in old bone.  
 387 The mean  $\Delta$ PPV and the statistical evaluation were calculated independently of the location of the bone (cortical and  
 388 trabecular). Statistical analysis showed that the effects of ALN and SrRan are not a function of the location (cortical and  
 389 trabecular) in old bone.  
 390

	mean $\Delta$ PPVs - old bone	ALN	SrRan	p-value
		Mean $\Delta$ PPV (95% CI)	Mean $\Delta$ PPV (95% CI)	
MINERAL	Mineral/Organic (Raman)	<b>+0.22 (+0.06 to +0.37)</b>	+0.11 (-0.04 to +0.27)	0.34
	Mineral/Organic (FTIRM)	+0.03 (-0.04 to +0.10)	+0.02 (-0.05 to +0.08)	0.76
	Carbonation-type-B $\times 10^{-3}$ (Raman)	-0.99 (-2.47 to +0.49)	+0.696 (-0.77 to +2.16)	0.11
	Carbonation $\times 10^{-3}$ (FTIRM)	-0.078 (-0.13 to +0.12)	-0.07 (-0.2 to +0.05)	0.48
	Crystallinity $\times 10^{-3}$ (Raman)	<b>+0.278 (+0.03 to +0.52)</b>	+0.041 (-0.2 to +0.29)	0.18
	Crystallinity $\times 10^{-3}$ (FTIRM)	+0.088 (-0.28 to +0.45)	-0.00043 (-0.37 to +0.37)	0.73
	Mineral maturity $\times 10^{-3}$ (FTIRM)	-12.15 (-74.38 to +50.07)	-24.63 (-86.85 to +37.60)	0.76
	ORGANIC	Hydro./Pro. $\times 10^{-3}$ (Raman)	<b>+8.33 (+0.59 to +16.07)<sup>c</sup></b>	<b>+30.77 (+23.06 to +38.47)<sup>a</sup></b>
Collagen maturity $\times 10^{-3}$ (Raman)		-2.28 (-14.79 to +10.23)	-18.88 (-31.3 to -6.45)	0.06
Collagen maturity $\times 10^{-3}$ (FTIRM)		-2.53 (-108.20 to +103.10)	+62.86 (-42.76 to +168.5)	0.38
PG/AmideIII $\times 10^{-3}$ (Raman)		+1.25 (-1.45 to +3.94)	<b>-13.55 (-16.25 to -10.85)<sup>a</sup></b>	<b>&lt;0.001</b>
NANO.	Elastic modulus (GPa)	+0.43 (-0.24 to +1.10)	+0.94 (+0.30 to +1.58)	0.27
	Tissue hardness (mPa)	+17.21 (-13.48 to +47.90)	+39.71 (+10.59 to +68.91)	0.29
	Working energy (pJ)	+75.71 (-92.89 to +244.32)	+218.50 (+58.11 to +378.89)	0.22
	Elastic energy (pJ)	+38.41 (-32.5 to +109.33)	+74.13 (+6.69 to +141.57)	0.46

391  
 392  $\Delta$ PPV corresponds to the difference in the PPV between M0 and M12 adjusted on the PPV value at M0. The p-value  
 393 corresponds to the comparison of the  $\Delta$ PPV between the two treatments.  
 394 The labels a:  $p < 0.001$ ; b:  $p < 0.01$ ; c:  $p < 0.05$  correspond to the comparison of the  $\Delta$ PPV with zero.

## 395 **6 Discussion:**

396 The aim of this study was to evaluate the differential effects of ALN and SrRan on bone quality in  
397 the short term at the ultrastructural level. The discussion is divided 2 sections. The first section discusses  
398 the results related to the new bone according to the mineral, organic matrix and nanomechanical  
399 properties. Similarly, the second section discusses the results related to old bone according to the  
400 mineral, organic matrix and nanomechanical properties.

### 401 **6.1 Effects of ALN and SrRan on the new bone**

402 **ALN and SrRan had differential effects on the mineral composition of newly formed bone after 12**  
403 **months of treatment.** After 12 months of ALN treatment, the newly formed bone showed that the  
404 mineral/organic ratio, crystallinity, and type-B carbonate contents (cortical) were increased. This is in  
405 agreement with previous studies involving bisphosphonate treatments and vibrational spectroscopic  
406 techniques. ALN treatment (for 2-3 years) progressively increased mineralization in iliac bone biopsies  
407 from women with PMOP [5, 6]. A similar study evaluated the effect of zoledronate (ZOL) and teriparatide  
408 (TPTD) on mineral composition in human iliac bone biopsies after 6 months of administration [35]. The  
409 recently formed bone (cortical and trabecular) under ZOL administration had increased mineral/organic  
410 and crystallinity value compared to TPTD at similar bone ages. The effect of ALN on mineral composition  
411 was also studied for long-term treatment by Raman and FTIRM spectroscopy. The mineral composition  
412 of recently formed bone was not different between 3 and 5 years of ALN administration [9]. Similarly,  
413 administration of ALN for up to 10 years results in minimal and transient bone composition compared to  
414 treatment for 5 years [8]. Our results show that mineral recently formed under ALN administration are  
415 more mineralized and organized (showing increased crystallinity) compared to the baseline. ALN has an  
416 effect on the mineral composition within the first year of treatment compared to long-term treatment,  
417 probably due to the duration of administration. This finding was also observed for BPs other than ALN  
418 [36, 37].

419 Concerning the biopsies treated with SrRan, EDX imaging analysis confirmed the presence of Sr<sup>2+</sup>  
420 in new bone, as previously reported [19, 38, 39]. The hydrated layer in new bone is larger than that in old  
421 bone [29]. The hydrated layer represents a pool of loosely bound ions that can be easily and rapidly  
422 exchanged with the surrounding environment. Thus, Sr<sup>2+</sup> was found in new bone because it is easily  
423 captured in the hydrated layer of the new bone. The implication of the hydrated layer in the  
424 physicochemical mechanism of Sr<sup>2+</sup> was also supported by the review of Marx *et al.*: "Sr treatment is  
425 more likely related to its interaction with collagen, water and the hydrated layer surrounding crystals,

426 rather than its ability to incorporate directly into HA" [13]. A decrease in the mineral/organic ratio after  
427 12 months of SrRan administration was observed by Raman spectroscopy but not by FTIRM. A previous  
428 study concluded that Raman spectroscopy is more sensitive than FTIRM to the mineral/organic ratio in  
429 bone samples [40]. This result could explain the significant decrease detected by Raman spectroscopy  
430 but not by FTIRM. Carbonation (Raman and FTIRM) and crystallinity were decreased in new bone. To  
431 date, the effect of SrRan on the carbonate content in human bone has not been reported. This effect was  
432 only reported in studies involving biomaterials, bone from animal models or cell cultures [41-43]. The  
433 incorporation of 1.5% Sr<sup>2+</sup> in synthetic hydroxyapatite (similar to the clinical dosage) did not change the  
434 composition of carbonate or the structure of the mineral [43]. The decrease in carbonation and  
435 crystallinity is not the result of the inclusion of Sr<sup>2+</sup> in the mineral. The presence of Sr<sup>2+</sup> reduced the  
436 crystal growth rates of hydroxyapatite in aqueous solution [44]. Thus, the presence of Sr<sup>2+</sup> in the  
437 hydrated layer might induce a delay in the maturation process of the mineral. Therefore, the lower  
438 mineral/organic (Raman), crystallinity, carbonation and mineral maturity (trabecular) observed in new  
439 bone at M12 might also be a consequence of delayed maturation. In cortical bone, conversely, mineral  
440 maturity was increased at M12 in new bone, but the reason for this is unclear. Doublier *et al.* assessed  
441 mineral changes in bone biopsies from women with PMOP treated with SrRan for 3 years [X-ray  
442 diffraction and selected area electron diffraction (SAED)] [16]; SAED allows the distinction between new  
443 and old bone packets. No modification of crystallinity in new bone was found. Thus, delayed maturation  
444 might be a transitory effect within the first year.

445         The design of our study took into account the location of the bone (cortical and trabecular). In  
446 the new bone, 2 of 15 PPVs were found to be location dependent. In the literature, there is no clear  
447 hypothesis about the differential effect of antiresorptive treatment on cortical and trabecular bone  
448 composition. In a 2-year ibandronate administration study, the results showed that cortical and  
449 trabecular bone reacted similarly to the effect of the treatment, as assessed by qBEI [45]. After 3 years of  
450 ALN administration, the mineral/organic increased only in cortical bone, as assessed by FTIRM [6]. It is  
451 noteworthy that neither previous study took into account the differences in bone age. Concerning the  
452 effect of SrRan, a study of 10 paired biopsies showed that the amount of Sr was higher in new trabecular  
453 bone than in new cortical bone, as assessed by XRD and SAED [16]. The difference in the bone turnover  
454 rate between cortical and trabecular bone was proposed to explain the result. The absence of a clear  
455 hypothesis might be related to the nature of the measured variables (labile or bulk compound). In our  
456 study, the changes in carbonation type B and mineral maturity depended on the location (cortical or  
457 trabecular). Carbonation type B evaluates the relative amount of carbonate type B in the mineral.

458 Carbonate is known to be a labile compound in bone mineral [46]. Mineral maturity (FTIRM) evaluates  
459 the relative amount of nonapatitic phosphate in bone mineral. Mineral maturity assesses the  
460 transformation of immature precursors of the hydrated layer into mature apatite [12]. Thus, both  
461 variables measure the relative amounts of labile compounds that are prone to be modified by the bone  
462 turnover rate. Both variables are functions of the location because the bone turnover rate is different  
463 between cortical and trabecular bone. The other variables (13/15) are not dependent on bone location  
464 because they evaluate the relative amount of compound founds in the bulk matrix (or much less labile),  
465 which are less prone to be modified by the bone turnover rate.

466 **The organic composition was not deeply modified by either treatment after 12 months in new bone.**

467 The organic matrix was assessed by FTIRM and Raman spectroscopy as 4 variables: collagen maturity  
468 (FTIRM and Raman spectroscopy), hydroxyproline/proline ratio (Raman) and PG/amide III (Raman).

469 After 12 months of ALN treatment, collagen maturity was not modified in new bone (FTIRM and  
470 Raman). The hydroxyproline/proline ratio was increased in new bone, revealing post-translational  
471 modifications in collagen. The PG content (PG/AmideIII) increased significantly in new bone after 12  
472 months of ALN. The relative PG content represents the noncollagenous compartment in bone. PGs play a  
473 role in regulating mineralization by affecting apatite nucleation and growth [47]. For the short-term  
474 treatment, the PG content was increased by ZOL compared to TPTD for 6 months of administration in  
475 newly formed bone (cortical and trabecular) [35]. Our results are in agreement with the short-term  
476 treatment. For long-term treatment of ALN, PG content in newly formed bone was not different between  
477 human bone biopsies after 3, 5 or 10 years [8, 9]. This apparent discrepancy between short- and long-  
478 term treatment might be due to the duration of administration. This result is not specific to ALN and was  
479 also observed for other BPs [36, 37].

480 After 12 months of SrRan treatment, the collagen matrix was not modified in new bone. The  
481 relative noncollagenous protein content (PG/AmideIII) was decreased in new bone. Recently, Marx *et al.*  
482 suggested that  $\text{Sr}^{2+}$  can form bonds with collagenous and noncollagenous proteins (such as  
483 proteoglycans) [13, 48]. The decrease in PG/amide III may result from the physicochemical effect of  $\text{Sr}^{2+}$   
484 on noncollagenous proteins.

485 **The nanomechanical properties are not modified by either treatment after 12 months in new bone.**

486 Previous studies have shown correlations between physicochemical parameters (Raman and FTIRM) and  
487 nanomechanical properties [49, 50]. Correlations were observed in animal models as a function of bone  
488 age. Mineral/organic, carbonation and crystallinity were found to increase with bone age. In our study,  
489 the mineral and organic composition were modified in new bone by ALN and SrRan. However, the bone

490 nanomechanical properties were not modified in new bone by either treatment. The modification of the  
491 composition of bone might not be enough to induce a significant effect on the nanomechanical  
492 properties. Modifications of nanomechanical properties have previously only been observed after long-  
493 term treatment with BPs [37, 51].

## 494 **6.2 Effects of ALN and SrRan on old bone**

495 **The mineral of old bone were modified by ALN but not by SrRan.** The statistical analysis showed that  
496 the effect of each treatment was the same on cortical and trabecular old bone (Table 4). After 12 months  
497 of ALN treatment, the mineral/organic (Raman) and crystallinity (Raman) increased in old bone. The  
498 amplitude of the increases in old bone is lower than those in new bone. Even if it is old bone, bone  
499 packets continued to mineralize slowly [10]. Therefore, the low increase is the result of the slow  
500 mineralization of old bone packets. The increase, detected by Raman spectroscopy and not by FTIRM, is  
501 certainly due to the sensitivity of Raman spectroscopy, as discussed in section 6.1 [40].

502 After 12 months of SrRan treatment, the mineral PPVs were not modified. The old bone has a thin  
503 hydrated layer (compared to new bone); thus,  $\text{Sr}^{2+}$  was not adsorbed in the old bone mineral. As a  
504 consequence, SrRan did not modify the mineral composition of old bone. This result supported the  
505 hypothesis of a physicochemical mechanism of action of  $\text{Sr}^{2+}$ , which is related to the hydrated layer [13].  
506 Moreover, it confirmed that modifications observed in mineral new bone were mainly related to the  
507 treatment and not artifacts of the embedding PMMA or long-term storage.

508 **The organic matrix is modified in old bone by ALN and SrRan after 12 months of administration.** After  
509 12 months of ALN treatment, collagen maturity and the PG content were not modified in old bone  
510 (FTIRM and Raman). The hydroxyproline/proline ratio was increased in ALN old bone, suggesting post-  
511 translational modifications in collagen. This increase might also be related to the increase in  
512 mineralization in old bone, as observed in the mineral old bone [52].

513 After 12 months of SrRan treatment, collagen maturity was not modified in old bone.  
514 Hydroxyproline/proline was increased in old bone, revealing post-translational modification of collagen.  
515 The increase induced by SrRan was higher than that induced by ALN. The relative noncollagenous protein  
516 content (PG/AmideIII) was decreased in old bone. The  $\text{Sr}^{2+}$  can form bonds with collagenous and  
517 noncollagenous proteins (such as proteoglycans) [13, 48]. According to Frankaer *et al.*,  $\text{Sr}^{2+}$  is fixed in  
518 both the mineral matrix and organic matrix [17]. These authors suggested the existence of inter- and  
519 intrafibrillar crosslinks in collagen. The increased hydroxyproline/proline and the decrease in PG/amide  
520 III may result from the physicochemical effect of  $\text{Sr}^{2+}$  on collagenous and noncollagenous proteins. This

521 strongly suggests a physicochemical mechanism of Sr<sup>2+</sup>, more than a biological effect, since the  
522 modifications occur in both new and old bone.

523 **The modifications of the physicochemical properties observed after 12 months of ALN or SrRan do not**  
524 **change the nanomechanical properties of old bone.** Similar to new bone, the bone nanomechanical  
525 properties were not modified by ALN in old bone. The effects on bone nanomechanical properties have  
526 been previously observed only after long-term treatments with ALN [37, 53].

527 The effects of Sr on bone nanomechanical properties have mainly been investigated in animal  
528 models [39, 54, 55]. These studies revealed that the bone became harder, stiffer and tougher after  
529 treatment for 6 and 52 weeks. However, in bone biopsies from patients treated with SrRan for 3 years,  
530 the indentation modulus of the SrRan group was not different from that of the placebo group at similar  
531 bone ages [15]. Finally, the modifications of the composition (mineral or organic) induced in old bone by  
532 either ALN or SrRan after 12 months were too small to induce a significant effect on the bone  
533 nanomechanical properties.

## 534 **7 Limitations and strengths**

535 First, the location of new and old bone was based on the anatomical location (FTIRM and  
536 nanoindentation) and ESEM images (Raman). Therefore, data were assessed from bone samples that did  
537 not strictly have the same age. This sampling procedure may induce variability in the results that is not  
538 related to the effect of the molecule. Second, the analyses were performed with 3 different techniques.  
539 The locations probed by each technique were not strictly the same. However, an effort was made to  
540 analyze similar bone areas between the three different techniques. Third, iliac crest biopsies at M0 and  
541 M12 were performed on the same subject. We made the assumption that modifications induced by the  
542 embedding procedure and the storage conditions were equal for all biopsies. Moreover, we compared  
543 the PPVs at comparable bone ages to minimize the effect of age. Therefore, if there were differences in  
544 PPVs between M0 and M12, these differences were mainly related to the effect of the treatment, and  
545 the other effects were minimized.

546 Despite these limitations, the main results are consistent with those in the literature. This study  
547 capitalizes on 2 main strengths. The analyses were performed with 60 paired biopsies, a larger number  
548 than previous studies involving analyses of bone quality at the molecular level. Finally, each biopsy was  
549 represented by 40 repeated measurements per spectroscopic technique and 5 repeated measurements  
550 for nanoindentation, a larger number of measurements than previous studies to produce robust results.



## 551 **8 Conclusions**

552 The analysis of 60 paired biopsies from postmenopausal women receiving either ALN or SrRan  
553 for 12 months showed that both compounds modulated bone quality, particularly in newly formed bone,  
554 but in different fashions. ALN modulated bone quality through its antiresorptive action. The bone formed  
555 during 12 months of ALN is characterized by increased mineral content, carbonation and apatite crystal  
556 size/perfection. The nanomechanical properties were not modified after 12 months, likely due to the low  
557 modification of the bone composition induced by ALN or SrRan for 12 months. SrRan decreased the  
558 crystallinity/perfection of the crystals and the carbonate content due to its physicochemical mechanism  
559 of action.  $\text{Sr}^{2+}$  cations are captured in the mineral matrix by the hydrated layer and modify the molecular  
560 composition of bone at the level of the bone structural unit. The collagenic and noncollagenic matrices  
561 are modified in old bone.  $\text{Sr}^{2+}$  might form bonds (crosslinks) with collagen and noncollagenous proteins.  
562 Our results showed that ALN and SrRan have differential effects on bone quality in relation to their  
563 mechanism of action. Both drugs modified the mineral and organic compositions in their own fashion  
564 within the first 12 months of treatment.

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## 570 **10 Conflicts of interest**

571 Dr Georges Boivin received grants and research support from Amgen, MSD, Servier. He was a consultant  
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575 other co-authors do not have conflicts of interest to declare that are related to the present study.

## 576 **11 CRediT authorship contribution statement**

577 All authors read and approved the final version of the manuscript. Conceptualization and design: GF, DF,  
578 GB, PA. Acquisition of the data: GF, DF, CP, PA. Analysis and interpretation of the data: all authors.  
579 Statistical analysis: GF, HB. Writing-original draft: GF, DF. Writing-review and editing: GF, DF, GB, BC.  
580 Revising and final approval: GF, DF, GB, BC.

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