

Effects of strontium ranelate administration on bisphosphonate-altered hydroxyapatite: Matrix incorporation of strontium is accompanied by changes in mineralization and microstructure

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ABSTRACT

Strontium ranelate (SR) is one therapeutic option for reducing risk of fracture in osteoporosis. The effects of SR treatment on hydroxyapatite (HA) previously altered by bisphosphonate (BP) administration remain to be established. Patients who have received long-term BP treatment and present with persistent high fracture risk are of particular interest. Paired iliac crest biopsies from 15 patients post-BP therapy were subjected to a baseline biopsy and a follow-up biopsy after treatment with 2 g SR day⁻¹ after either 6 months ($n = 5$) or 12 months ($n = 10$). Dual energy X-ray absorptiometry scans, serum parameters and biochemical markers were obtained. Quantitative backscattered electron imaging and energy-dispersive X-ray analyses combined with micro-X-ray fluorescence determinations were performed to observe any mineralization changes. Static 2-D histomorphometry was carried out to evaluate cellular and structural indices. After 6 months of SR treatment, increases in osteoid surface and strontium content were observed, but no other indices showed significant change. After 12 months of SR treatment, there was a significant increase in bone volume and trabecular thickness, and further increases in strontium content and backscattered signal intensity. These structural changes were accompanied by increased numbers of osteoblasts and increased osteoid surface and volume. Additionally, low bone resorption, as measured by beta-cross-laps, and a low number of osteoclasts were observed. SR treatment led to increased strontium content within the BP-HA nanocomposites and to increased osteoid indices and bone volume, which is indicative of newly formed bone, while osteoclasts were still suppressed. These data points suggest that SR might be considered as a therapeutic option for patients following long-term BP treatment.

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1. Introduction

Bone quality is considered to be the total sum of characteristics that influence resistance to fracture. A well-regulated interplay of bone's structural and material properties preserves bone strength to avoid fractures. In this context, disorders at the structural level concerning bone size, geometry and microarchitecture are known to affect bone strength negatively, whereas at the material level, redistributions of collagen and mineral content impair bone tough-

ness [1–5]. These changes frequently become apparent in post-menopausal osteoporosis, which is the most common metabolic bone disease and is characterized by an imbalance between bone resorption and bone formation in the physiological process of bone remodeling [6]. Osteoporotic fractures, especially fractures of the wrist, the spine and the hip, are a consequence of a reduction in bone mass, the deterioration of structural integrity and additional changes in mineral content and/or distribution [1,3].

Currently, several agents are available to address patients' specific medical needs for osteoporosis treatment. Two main types of drugs are applied: anti-resorptive drugs (e.g., bisphosphonates, hormone replacement therapy and selective estrogen-receptor modulators), which slow the progression of bone loss [7–9], and agents that stimulate bone formation (e.g., parathyroid hormone fragments) to support increases in bone mass [10]. Recently, strontium

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ronelate (SR) has been considered as an additional alternative treatment for osteoporosis, because it has been shown to reduce the risk of vertebral fracture and hip fracture [11–13]. The latter effects of SR administration are considered to result from a mode of action involving both the stimulation of bone formation and decreases in bone resorption [6,13–16]. While approved medications have demonstrated efficacy in reducing fracture risk in osteoporosis patients, several details of these treatments remain to be established. These open questions include the optimal duration of treatment intervals and appropriate combinations of different therapy strategies, because prior bisphosphonate (BP) exposure may cause blunting of the SR properties [17]. To date, these issues have been poorly investigated, and only one study has focused on the volumetric bone mineral density (DXA-BMD) and bone turnover markers in response to continuative SR treatment [17]. Accordingly, the present paper focuses on the bone matrix properties in terms of the osseous changes in microstructure, mineralization and cellular characteristics of patients who have been on BP therapy for years, but have switched to SR treatment owing to residual fracture risk.

Previously administered BP accumulates in the bone tissue and alter the hydroxyapatite (HA). This accumulation occurs because the phosphonate groups of the BP bind to Ca^{2+} ions in HA crystals. The aim of this study is to determine whether BP-modified HA responds differently to subsequent SR therapy with regard to bone-HA properties. In order to gain insight into the effects of SR therapy following long-term BP treatment, paired iliac crest biopsies from 15 patients were obtained from the Hamburg Bone Register. Thus, the effects of SR administration following BP treatment were studied at the mineral and structural level by microanalyses, evaluations of the bone mineral density distribution (BMDD) and 2-D histomorphometry. This study delineates the changes in cellular and structural parameters as well as in mineral content and mineral distribution after SR treatment in patients previously treated with BP.

2. Materials and methods

2.1. Cohort selection criteria and patients' characteristics

Bone biopsies were obtained from 15 female osteoporotic patients within the same age range who had been on long-term BP treatment. The patients were pre-treated with alendronate at either 10 mg day^{-1} or 70 mg week^{-1} . Patients with persistent high fracture risk suffering from new vertebral fractures or a BMD decline during adequate BP treatment were regarded as suitable for alternative osteoporosis treatment, and therapy was therefore changed from BP to SR. In addition, two patients showed BP intolerance due to gastrointestinal complaints.

The inclusion criteria that determined patient eligibility for the study were as follows. All participants were postmenopausal and >55 years old and were required to have a minimum of one osteoporotic vertebral fracture and a lumbar spine or total hip BMD T-score of ≤ -2.5 prior to the initiation of BP therapy. Patients were considered BP non-responders when BMD measurements were documented with >3.5% decline per year at the hip or lumbar spine or new fragility fractures occurred following at least 1 year of BP treatment.

Subjects with any of the following conditions were ineligible (exclusion criteria): subjects who were taking medication, other than BP and SR, which affects mineral homeostasis (calcitonin, glucocorticoids, sodium fluoride); individuals who suffered from cancer, renal diseases, primary hyperparathyroidism, gastrointestinal disease, Paget's disease, alcohol abuse, illicit drug use or showed any other symptoms of bone diseases apart from postmenopausal osteoporosis.

Compliance was measured by evaluation of the bone strontium content detected in all biopsy samples and by a compliance questionnaire for each visit. In accordance with local ethical standards, informed consent was obtained from all patients, and the study was approved by the Medical Association Ethics Committee of the State of Berlin. Moreover, all 15 patients included gave written informed consent to undergo two biopsies: one at baseline when their BP treatment (≈ 32 months) was terminated, and one after 6 ($n = 5$; group I) or 12 months ($n = 10$; group II) of treatment with 2 g SR day^{-1} during ongoing adapted standard calcium and vitamin D supplementation. Each of the bone biopsies was retrieved from the dorsal iliac crest by Jamshidi biopsy technique [18]. All biopsies were performed at the same clinical department by one of the authors (JS, Immanuel-Hospital Berlin-Wannsee, Berlin, Germany) from alternating corresponding sides of the pelvis at each visit. JS used an 8-gauge Jamshidi needle with an inner diameter of 3 mm for the biopsies (Cardinal Health, McGaw Park, IL, USA). A local infiltration of the exterior periosteum with lidocaine hydrochloride (1%, 20 ml, without adrenaline) ensured sufficient anesthesia of the iliac wing. The biopsies meet the standard required for histomorphometry because the length of the biopsies averages 2 cm (0.79 in.). Thus, the bone volume of Jamshidi biopsies for histomorphometry equates to the bone volume of common transiliacal Bordier biopsies without the limiting factor of bilateral subcortical bone heterogeneity. Parameters assessing trabecular interconnection are not included in the static histomorphometric analysis in this study. BMD measurements were performed by dual energy X-ray absorptiometry (DXA) using the GE Healthcare Lunar Prodigy DXA Scan (Madison, USA). The parameter T-score was measured to give interpretation of the BMD results. It shows the discrepancy of the patient's BMD value from the mean BMD for a young adult healthy population in units of the population standard deviation (SD). A T-score > -1 is considered normal, a T-score between -1 and -2.5 is classified as osteopenia, and a T-score < -2.5 is defined as osteoporosis. The lumbar spine (L2–L4 vertebrae) and the left total hip (neck, Ward's triangle and greater trochanter) were scanned using the standard protocols provided by the manufacturer before and after SR treatment. Moreover, the evaluation of adjusted BMD values due to assumed Sr deposition was performed on the basis of the calculations given by Blake et al. for GE Lunar systems [19–21]. The latter correction was done while considering the specific strontium content in the individual patients, because the strontium content was measured in all bone biopsies.

2.2. Undecalcified preparation of the specimens

The plastic embedding after undecalcified preparation was accomplished as described previously to allow artifact-free preparation of the bone tissue to preserve the bone matrix and cellular structures [22]. After the expiration of the polymerization, the polymethyl-methacrylate (PMMA) embedded specimens had a cylindrical block shape, which is appropriate for microtome cutting. The sections were cut in the longitudinal plane on a rotation microtome (Cut 4060E, MicroTech, Munich, Germany). Cut sections with a thickness of $\sim 4 \mu\text{m}$ were stained according to standard procedures (von Kossa, Giemsa and Goldner) as described [22]. Furthermore, the PMMA blocks with the incorporated bone specimens were processed via Donath's grinding technique [23] to coplanar polished specimens. This enabled analysis of the elemental composition (strontium content) using energy-dispersive X-ray analysis (EDS) combined with micro-X-ray fluorescence (μXRF), and quantitative backscattered electron imaging (qBEI) for the analysis of BMDD.

2.3. EDS/ μ XRF analyses

Non-destructive microanalysis was carried out by EDS (EDAX, DX-4, Mahwah, NJ) and μ XRF (IFG, IMOXS, Berlin, Germany) analysis on the embedded undecalcified coplanar polished bone biopsies. By combining EDS and μ XRF, the detection limit of heavier elements ($z = 11$) can be improved by factors of 20–50 down to a range of <50 ppm, due to a reduced spectral background [24,25]. In order to minimize the effects of variously aged bone packets and remodeled bone packets, the X-ray and electron beam, were exclusively focused on pronounced large-scaled trabecular nodes. Therefore, comparisons of the quantified concentrations were related solely to the center of nodes with a transverse section >100 μ m. The strontium (Sr) content in bone biopsies, as well as the elemental composition of the bone matrix, including trace metals, was evaluated in weight per cent (wt.%) via software that enabled combined EDS/ μ XRF quantification (IMOXS Quant 2.10, IFG, Berlin, Germany).

2.4. Clinical laboratory analysis of serum parameters and biochemical markers

Morning fasting blood samples were obtained from all patients at three different times: (i) after alendronate treatment (pre-SR); (ii) after 6 months on SR (group I); or (iii) after 12 months on SR treatment (group II). All samples were taken during adequate calcium + vitamin D substitution. The blood samples served for evaluation of serum parameters and biochemical markers such as calcium (Ca, mmol L⁻¹), phosphorus (P, mmol L⁻¹), creatinine (μ mol L⁻¹), total alkaline phosphatase activity (ALP, U L⁻¹), bone alkaline phosphatase (bALP, μ g L⁻¹) and beta cross-laps (β -x-laps, pg nl⁻¹). The latter samples were obtained from all patients on the days of the biopsies. The samples were taken 24 h after the last BP or SR and Ca/vitamin D administration. Each blood sample was centrifuged, and the separated serum was stored at -30 °C.

2.5. Static structural and cellular 2-D histomorphometry

Toluidine blue stained, undecalcified 4- μ m histological sections were used for 2-D histomorphometric assessment according to the ASBMR histomorphometry nomenclature guidelines [26]. The ratio of bone volume to tissue volume (BV/TV, %), the trabecular thickness (Tb.Th., μ m), the ratio of osteoid volume to bone volume (OV/BV, %), the ratio of osteoid surface to bone surface (OS/BS, %), the ratio of the number of osteoblasts to the bone perimeter (NOB/BPm, 1 mm⁻¹) and the ratio of the number of osteoclasts to the bone perimeter (NOc/BPm, 1 mm⁻¹) were determined by image analysis (OsteoMeasure Analysis System, Osteometrics, Atlanta, GA).

2.6. Bone mineral density distribution

The evaluation protocol of quantitative backscattered signal intensities is based on the work of other groups and has been described previously [3,27]. The scanning electron microscope (LEO 435 VP, LEO Electron Microscopy Ltd., Cambridge, UK) was operated at 15 kV and 665 pA at a constant working distance (BSE Detector, Type 202, K.E. Developments Ltd., Cambridge, UK). A pixel size of 3 μ m was chosen following the recommendation of Roschger et al. [28]. The quantification was standardized by the analysis of synthetic HA. Seven HA samples with progressively increasing Ca/P ratios (D.O.T. Medical Solutions, Rostock, Germany) were quantified with EDS and qBEI to create a calibration curve. A highly linear relationship between backscattered electron imaging gray values and the calcium content (Ca wt.%) has been reported in several studies [29–31]. The linear dependence ($r = 0.98$) of the

evaluated HA gray values in terms of the respective calcium concentration of the HA samples are used to calibrate the method. Because of the assumed deposition of Sr into the mineralized matrix, the backscattering probability changes. Backscattering of electrons in a single collision with atomic electrons is impossible. Therefore, it is assumed that this occurs only by nuclear backscattering. The probability for this process is, at first approximation, proportional to the effective atomic number squared (Z_{eff}^2) of the mineral content (calcium hydroxyapatite = CaHA and strontium-containing hydroxyapatite = SrHA, respectively). If CaHA is partly replaced by SrHA, this will result in a correction of the backscattered electron intensities according to Eq. (1).

Adjusted Gray Level(aGL)

$$= \text{Gray Level(GL)} \times \frac{Z_{\text{eff}}^2[\text{CaHA}]}{Z_{\text{eff}}^2[(1 - \alpha)\text{CaHA} + \alpha \cdot \text{SrHA}]} \quad (1)$$

where α is the molar fraction of strontium, which is substituted for Ca, and Z_{eff}^2 is the average Z^2 of the corresponding mineral. In this approximation, backscattering from soft tissue is neglected owing to its low atomic numbers. In the case of Sr deposition, in addition to unchanged CaHA content (Sr adsorption), the correction factor for backscattering will increase insignificantly.

2.7. Statistical analysis

Continuously variable data was subjected to the Shapiro–Wilk test to define distribution. In the case of normally distributed data, the differences in investigated properties before and after SR treatment were assessed by paired *t*-test, while for non-parametric data the paired Wilcoxon rank sum test was used. Values of $P \leq 0.05$ were considered significant.

3. Results

3.1. Baseline characteristics

The age range of 15 female osteoporotic patients was 65.27 \pm 8.33 years, and the administration period of alendronate was, on average, 32 months, with longer periods of calcium and vitamin D supplementation. In addition to measured DXA T-scores, the documentation of multiple fractures (1.87 \pm 1.6) proved manifested osteoporosis in all patients studied. Serum parameters and biochemical bone markers varied within the range of tolerance (Table 1).

3.2. DXA, serum parameters and biochemical markers after SR treatment

Group I T-scores, without and with adjustment due to Sr deposition, revealed no significant differences between the baseline and 6 months' SR treatment. In group II, after 12 months of treatment, SR significantly increased bone mineral density at the femoral neck (baseline -2.26 \pm 1.15 (T-score) vs 12 months -1.80 \pm 0.61 (T-score); $P = 0.01$) and spine levels (baseline -3.21 \pm 0.72 (T-score) vs 12 months -2.9 \pm 0.93 (T-score); $P = 0.02$). However, Sr adjusted T-scores revealed no significant differences after 12 months' SR treatment at the femoral neck or the spine levels, compared with the baseline (Table 2). Serum parameters of calcium, phosphorus and creatinine measured at the baseline and after 6 or 12 months of SR administration remained in a normal range and without significant changes. After 6 months of treatment with SR (group I), there was no detectable change in the degree of total alkaline phosphatase activity and bALP activity. In contrast, after 12 months of SR therapy, there was a significant increase in ALP (baseline 80.1 \pm 22.32 (U L⁻¹) vs 12 months 89 \pm 22.6 (U L⁻¹);

Table 1
Baseline characteristics of the bone biopsy population.

n = 15	Age (yr)	Vert. Fx (No.)	T-score Hip	T-score L2–L4	BP (mo.)	Ca + Vit. (mo.)
Mean	65.27	1.87	−2.18	−3.12	32.33	41.13
SD	±8.33	±1.60	±1.02	±0.71	±15.5	±31.89
n = 15	Ca (mmol L ^{−1})	P (mmol L ^{−1})	Crea. (μmol L ^{−1})	β-x-laps (pg ml ^{−1})	ALP (U L ^{−1})	bALP (μg L ^{−1})
Mean	2.35	1.21	69.87	238.82	83.13	12.45
SD	±0.11	±0.23	±9.51	±153.5	±21.79	±2.82

Values are presented as mean ± S.D. (standard deviation); Vert. Fx: number of vertebral fractures; T-score: represents number of standard deviations from mean bone mineral density (BMD) of a young adult reference population; BP (mo.): bisphosphonate treatment in months; Ca + Vit. D (mo.): calcium + vitamin D supplementation in months; Ca: calcium; P: phosphorus, Crea.: creatinine; β-x-laps: beta-cross-laps (type I collagen degradation fragments (β-CTX)); ALP: alkaline phosphatase; bALP: bone specific alkaline phosphatase.

Table 2
Changes in DXA, serum electrolytes and biochemical markers after 12 mo. strontium ranelate.

n = 10	Baseline	SR (12 mo.)	P
DXA Hip (T-score)	−2.26 ± 1.15	Regular	−1.8 ± 0.61 *
		Adjusted	−2.35 ± 0.57 N.S.
DXA L2–L4 (T-score)	−3.21 ± 0.72	Regular	−2.9 ± 0.93 *
		Adjusted	−3.33 ± 0.74 N.S.
Ca (mmol L ^{−1})	2.37 ± 0.1	2.39 ± 0.07	N.S.
P (mmol L ^{−1})	1.15 ± 0.23	1.168 ± 0.27	N.S.
Creatinine (μmol L ^{−1})	71.57 ± 10.25	71.64 ± 8.17	N.S.
β-x-laps (pg ml ^{−1})	227.5 ± 156.9	263.8 ± 124.3	N.S.
ALP (U L ^{−1})	80.1 ± 22.32	89 ± 22.6	*
bALP (μg L ^{−1})	12.25 ± 1.84	14.57 ± 3.87	*

Values are presented as mean ± S.D. (standard deviation); DXA Hip and L2–L4 T-score values are presented as mean ± S.D. (standard deviation) of regular (non-adjusted) BMD in g HA/cm² as well as adjusted BMD in g HA/cm² based on a modified formula given by Blake & Fogelman [19,20]; Ca: calcium; P: phosphorus, Crea.: creatinine; β-x-laps: beta-cross-laps (type I collagen degradation fragments (β-CTX)); ALP: alkaline phosphatase; bALP: bone specific alkaline phosphatase; N.S. = not significant; * = $p < 0.05$.

$P = 0.05$) and bALP activity (baseline 12.25 ± 1.84 (μg L^{−1}) vs 12 months 14.57 ± 3.87 (μg L^{−1}); $P = 0.05$). Changes in bone resorption, as assessed by measurement of β-x-laps, remained non-significant and low overall (Table 2).

3.3. Quantification of Sr content using EDS/μXRF microanalyses

The microanalysis spectra obtained reveal the elemental composition of the specimens. Distinctive element peaks predominantly from calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), potassium (K), fluoride (F), chlorine (Cl), strontium (Sr), zinc (Zn) and copper (Cu) are quantifiable. Baseline biopsies had a mean Sr content of 0.11 ± 0.08 wt.% Sr (group I) and 0.15 ± 0.1 wt.% Sr (group II). Post-treatment, the analysis following SR therapy revealed considerable peaks in the spectra, indicating an increase in Sr content within the trabecular nodes of bone tissue. The 6-month administration of SR, therefore, showed a significant increase in Sr bone concentration (baseline 0.11 ± 0.08 (wt.% Sr) vs 6 months 0.40 ± 0.25 (wt.% Sr); $P = 0.03$) (Fig. 1A–C). In group II, the administration of SR for 12 months resulted in a distinct increase in Sr content, as the differences in comparison with the untreated phase proved to be significant (baseline 0.15 ± 0.1 (wt.% Sr) vs 12 months 0.80 ± 0.52 (wt.% Sr); $P = 0.003$) (Fig. 1D–F).

3.4. Bone mineral density distribution

Evaluation of the backscattered signal intensities obtained from the mineralized phase of bone tissue indicated the degree of mineralization among the investigated groups. The comparison of evaluated gray levels revealed no significant differences in group I (baseline 22.11 ± 3.00 (wt.% Ca) vs 6 months 21.71 ± 1.18 (wt.%

Ca + Sr)) (Fig. 2A; Table 3). In contrast, group II demonstrated an increase in backscattered signal intensities (baseline 21.63 ± 1.84 (wt.% Ca) vs 12 months 22.91 ± 2.55 (wt.% Ca + Sr); $P = 0.03$) (Fig. 2B; Table 3). After correcting for the substituted Sr using the adjustment equation (Eq. (1)), there were only non-significant changes of the mean wt.% Ca in both groups.

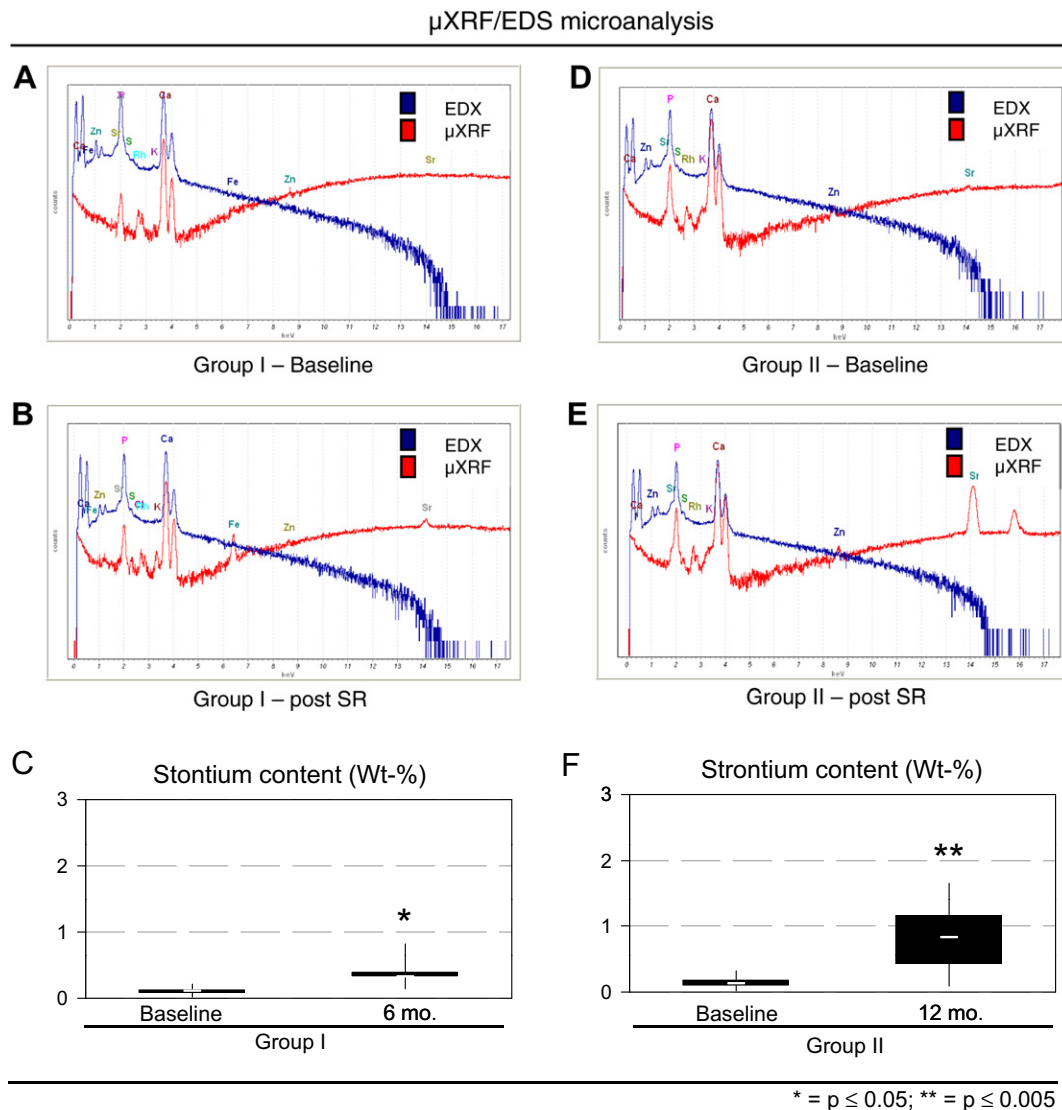
3.5. 2-D histomorphometry

The ratio of bone volume to tissue volume (BV/TV, %), did not show significant differences when compared with the baseline in group I (6 months treatment with SR). In contrast, BV/TV was significantly increased in the biopsies of group II after 12 months of SR treatment (Fig. 3A and B; Table 4). Similarly, Tb.Th. was unchanged in group I, whereas a significant increase in trabecular thickness was detected in group II (Fig. 3A and B; Table 4). Further evaluations of OV/BV revealed no significant differences in group I. In group II, OV/BV was significantly increased after 12 months' SR treatment. Significant changes in OS/BS occurred in group I and in group II. In group I, the NOb/BPm remained unchanged, whereas group II demonstrated a significant increase in NOb/BPm. The NOc/BPm did not change significantly in both group I and in group II (Table 4).

4. Discussion

Microanalytical and histomorphometric assessment of the bone biopsies revealed that 12 months of treatment with SR following long-term BP therapy leads to significant strontium deposition in bone, which affects DXA-BMD and qBEI measurements. However, there were no signs of mineralization defects or other detrimental alterations of the mineralized bone matrix. Furthermore, a significant increase in osteoid and osteoblast indices is indicative of newly formed bone in the face of persistent low bone resorption. These findings are supported by a concomitant increase in BV/TV as assessed by 2-D histomorphometry but, in accordance with previous observations in osteoporosis patients, without anti-resorptive pre-treatment [6,32].

In a previous study on patients with SR treatment, Arlot et al. demonstrated the effects of SR in 2-D histomorphometric and 3-D microcomputer tomography (3-D-μCT) analyses of biopsies from 133 patients. 2-D histomorphometry showed significant increases in mineral apposition rate and significant decreases in osteoid thickness, and the authors suggest that this might indicate an activation of osteoblasts. In that study, BV/TV assessed by 3-D-μCT was significantly higher in patients treated with SR for 36 months compared with those treated with a placebo [6]. Furthermore, since the patients studied by Arlot and co-workers had not received previous anti-resorptive treatment, the current work may contribute a better understanding of the mechanism of presumed early osteoblastic stimulation following SR treatment [6]. The present



* = $p \leq 0.05$; ** = $p \leq 0.005$

Fig. 1. (A) An example of a baseline spectrum from group I, indicating no distinct peak for Sr. (B) Microanalysis spectrum indicating a Sr peak after 6 months of treatment. (C) An example of a baseline spectrum from group II with no pronounced Sr peak. (D) A representative spectrum with a pronounced Sr peak after 12 months of SR treatment. (E, F) Significantly elevated Sr concentration after 6 months of treatment and significantly increased Sr concentration in bone tissue after 12 months of SR treatment.

study suggests that osteoblasts may be activated, since surrogate parameters such as bALP increased after 12 months of SR treatment, which is morphologically confirmed by significant increases in osteoid surface after only 6 months of treatment and by a significant increase in osteoid surface and volume after 12 months of treatment. The latter increase in osteoid indices is not an indicator of a mineralization defect, because all values are clearly in the physiological range [33], but rather indicates an activation of osteoblasts, which is confirmed by a significant increase in osteoblast indices. Thus, the effect of prior BP therapy, which is known to reduce bone turnover [34,35], did not inhibit recurrent rises in bone forming activity shortly following the cessation of anti-resorptive treatment. This is also supported by the study of Middleton et al., who reported that, after the discontinuation of BP and switching to SR, a significant suppression of bone turnover remains for 3–6 months [17]. However, Hwang et al. showed that, without prior BP treatment, bone formation markers were already increased at 6 months and also at 12 months, owing to SR treatment [36]. The observed effects on osteoblasts and osteoclasts measured in this study may therefore confirm previous findings of SR's mode of action [16,37,38], but another possible explanation

for the current findings could be that vanishing BP effects are operative [39,40]. The reported increase in bone volume and trabecular thickness in this study may indeed reinforce a possible dual mode of action of SR. Defined by given bone histomorphometry equations, the bone volume per tissue volume represents mineralized bone plus the unmineralized osteoid per tissue volume. Therefore, increases in osteoid parameter also display an increase in BV/TV and trabecular thickness. This may be interpreted as an anabolic effect of SR once the osteoid becomes mineralized. However, this progress is challenging to prove; a study focusing on bone biopsies after longer treatment periods will be necessary.

Increases in BMD values measured by DXA at the hip and lumbar vertebrae after 12 months of SR treatment may be attributable to improvements in bone mass and/or mineral density on the tissue level. Also, in a 12-month study of patients treated with SR without prior BP treatment, significant increases in DXA-BMD were shown [36]. However, the deposition of Sr with a higher atomic number than Ca in the bone matrix may lead to BMD overestimation of the dependence of the strontium uptake in bone [19–21]. The evaluated overestimation of $\sim 10\%$ BMD (g cm^{-2}) in the group of patients treated for 12 months with a median strontium

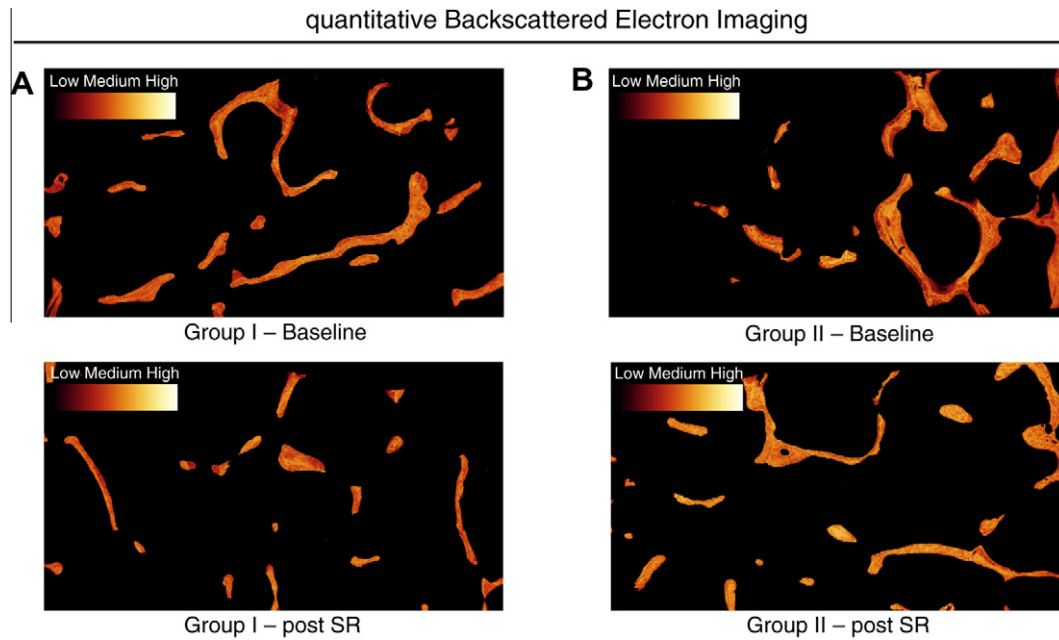


Fig. 2. BMDD from backscattered electron images (BSE, 25 \times). The pixel brightness indicates the degree of mineralization. Thus, greater brightness indicates higher Ca content, and lesser brightness indicates lower Ca content. (A) Levels of Sr incorporation after 6 months of treatment do not result in elevated pixel brightness. (B) Administration of SR for 12 months leads to a shift in pixel brightness when compared with the baseline.

Table 3
Bone mineral density distribution.

BMDD	Group I (baseline)	Group I (6 mo. SR)	<i>P</i>	Group II (baseline)	Group II (12 mo. SR)	<i>P</i>
wt.%	(Ca) 22.11 \pm 3.00	(Ca + Sr) 21.71 \pm 1.18 Adjusted (Ca) 21.72 \pm 1.03	N.S. N.S.	(Ca) 21.63 \pm 1.84	(Ca + Sr) 22.91 \pm 2.55 Adjusted (Ca) 21.77 \pm 2.73	* N.S.

Values are presented as mean \pm SD. N.S. = Not significant; * = $p \leq 0.05$.

concentration of 0.80 wt.% corresponds to the values described by Blake and Fogelman as well as by Pors-Nielsen et al. for 1 mol mol. $^{-1}$ Sr [19–21,41]. Consequently, the adjusted T-scores corrected for Sr incorporation suggest that conventional BMD increases may indeed be predominantly the result of Sr incorporation and do not represent real increases in mineralized bone mass in the patients studied. Meanwhile, however, although different algorithms for adjusting the BMD in patients on SR therapy have been proposed, none is validated [20,42]. There are differences in strontium incorporation in bone independent of both the current mineralization status and the skeletal site. In this connection, measurements of strontium content in the femoral neck could not be determined and thus have been adapted for an approximation from the microanalysis of the iliac crest biopsies. Moreover, strontium is incorporated primarily during primary mineralization following SR treatment rather than in older bone formed before treatment initiation [42,43]. The current bone turnover status will therefore most likely render incorporation and distribution of bone strontium content. Hence, BMD adjustment algorithms are complex and include numerous assumptions, as it is impossible to assess and consider individual patients' 3-D strontium distribution related to the spine and hip levels. However, in non-adjusted BMD, the greater changes may be of direct clinical relevance, since positive changes in BMD may serve as an indication of patient response to therapy [42]. In addition, groups of patients with pronounced increases in measured (unadjusted) BMD were demonstrated to have predominant fracture risk reduction [42,44]. Therefore, monitoring of unadjusted BMD in individual SR-treated patients with discernable increases in BMD could be correlated with antifracture efficacy [42,44].

Several studies showed that pharmaceutical agents may have remarkable effects on the degree of mineralization, and also the crystal properties, of the bone matrix [45,46]. As in previous SR studies, no evidence was found of abnormal bone formation, woven bone or any detrimental mineralization defects under SR [6]. In addition, similarly to previous studies, it is confirmed that Sr is deposited primarily in newly formed bone, whereas the Sr concentration in older, higher mineralized bone packets [43,45,47–49] was lower, since the age of trabecular nodes can be characterized by backscattered signal intensities. However, regarding the effect of Sr incorporation into the bone matrix, the correction factor for quantitative electron backscattering is small compared with the X-ray transmission (DXA) reduction. This is because the X-ray absorption is considerably more dependent on the atomic number than backscattering is. In contrast to the findings described by Farlay et al., who reported a preservation of the degree of bone mineralization in SR-treated monkeys [47], the present study found a slight increase in mineralization following 12 months of SR treatment. Again, one has to take into account that Sr is now a part of the mineralized bone. Li et al. reported a shift towards the higher mineralized range by the evaluation of backscattered signal intensities [49]. Independent of the mechanism of strontium uptake, whether Ca is substituted by Sr crystals [47,49] or Sr is adsorbed onto the mineral surface [43], the element Sr, with an approximately twofold higher atomic weight (Sr = 87.62 u) than Ca (Ca = 40.08 u), will increase backscattered signal intensities. However, the applied adjustment of backscattered signal intensities regarding possible Ca substitution by Sr enables the evaluation of gray level change due to SrHA formation. The part of Sr that is mainly adsorbed onto the mineral surface of newly formed bone

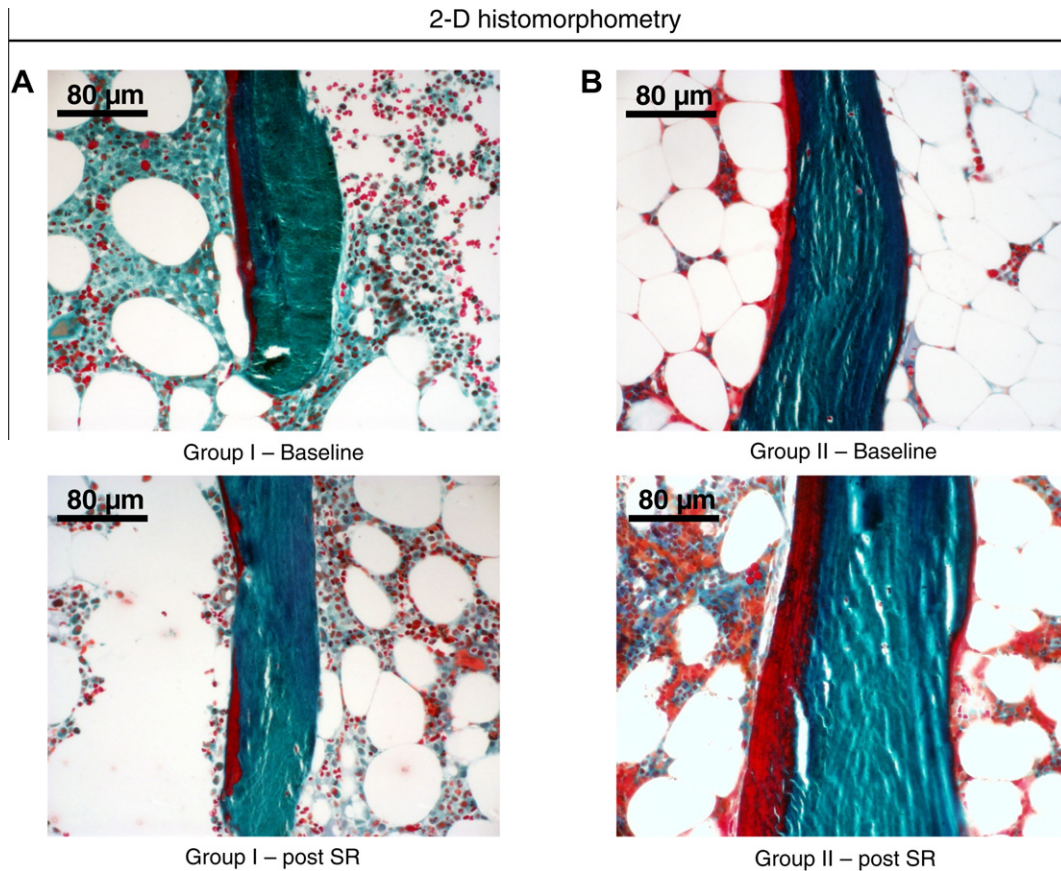


Fig. 3. 2-D histomorphometry (Goldner, 200 \times). (A) Trabeculae before and after 6 months of SR treatment. (B) Thickening of the trabeculae and pronounced osteoid formation is evident in group II following 12 months of SR administration with significant increases in the BV/TV, Tb.Th., and osteoid parameters. (A, B) OV/BV increased significantly after 12 months' SR treatment, whereas significant increases in OS/BS are evident after both 6 months and 12 months of SR treatment.

Table 4

2-D Histomorphometry: structural indices.

2-D data	Group I (baseline)	Group I (6 mo. SR)	<i>P</i>	Group II (baseline)	Group II (12 mo. SR)	<i>P</i>
BV/TV (%)	8.16 \pm 5.06	7.84 \pm 2.49	N.S.	8.53 \pm 2.14	11.12 \pm 3.79	*
Tb.Th. (μ m)	91.71 \pm 11.5	89.38 \pm 7.91	N.S.	89.99 \pm 10.96	99.39 \pm 9.89	*
OV/BV (%)	0.33 \pm 0.29	0.46 \pm 0.45	N.S.	0.49 \pm 0.63	1.2 \pm 1.18	*
OS/BS (%)	7.32 \pm 5.3	13.01 \pm 9.07	*	7.97 \pm 5.60	12.04 \pm 6.81	*
NOb/BPm (1 mm ⁻¹)	0.43 \pm 0.44	0.24 \pm 0.29	N.S.	0.32 \pm 0.39	0.58 \pm 0.45	*
NOc/BPm (1 mm ⁻¹)	0.02 \pm 0.02	0.01 \pm 0.01	N.S.	0.04 \pm 0.07	0.01 \pm 0.02	N.S.

Values are presented as mean \pm S.D. (standard deviation). N.S. = NOT significant; * = $p \leq 0.05$.

plays a minor role in these calculations, because Sr concentrations were measured in mature trabecular nodes. This adjustment showed no significant changes in wt.% Ca compared with the baseline for the group treated for 6 months and the group treated for 12 months. Thus, Ca content does not decrease, and bone maintains the Ca content on the level detected directly after BP treatment. However, mineralized tissue with incorporated Sr makes the bone heavier compared with the baseline. Any effects resulting from heavier bones due to Sr incorporation concerning bone quality evaluation remain uncertain at present. Fuchs et al. reported no significant improvements in the mechanical properties of strontium-containing lumbar spine in rat bones [50], whereas Bain et al. [51] and Ammann et al. [52] reported that a significant increase in load and energy is required to induce lamellar deformation in rat bones treated with SR.

In order to improve methodological immanent detection limits, EDS analyses were combined with μ XRF analyses for the detection of Sr *K*-alpha lines (to avoid interference between different *L*-alpha lines) using low, surface preserving excitation voltage and making

use of the higher fluorescent yield of *K*-alpha lines. Accordingly, excitation combination of lighter elements by EDS [53], as well as of heavier elements due to the trace element range by μ XRF, may provide a more precise quantification for Sr content. In bone samples, the depth of the X-ray penetration is several orders of magnitude larger than the depth of the electron beam, which results in larger excited volumes and thus leads to better statistics and reliability of the measured intensities. However, designations of Sr content in terms of bone mineral density remain difficult because of the non-appearance of defined measuring volumes. Since EDS microanalyses are restricted to the sample surface, while μ XRF gain information from different sample depths, non-destructive investigations of histological slices with a defined thickness, similar to that used for proton induced X-ray emission (PIXE), may provide further detailed information [54]. The effects of Sr content on bone mineral density remain of particular interest for DXA calibrations [19–21]. Boivin et al. reported gradient increases of Sr uptake between treatment periods of 2 and 3 years [43]. In accordance with their findings, the present authors also analyzed gradient

increases of Sr concentration within the mineralized tissue between treatment periods of 6 and 12 months. Comparisons between the evaluated Sr concentrations and the values reported in previous studies proved to be difficult owing to different microanalysis techniques and varying selection of measuring points [43,45, 48–50]. Further accomplishments of microanalyses focusing on advanced treatment periods may be beneficial, since precise information about the timeline of bone matrix saturation with Sr is still missing.

This is the first histomorphometric and microanalytic analysis of the changes at the bone tissue level in a patient cohort that has received SR after long-term BP treatment. As such, these results are of direct clinical importance. One has to note, however, that there are also at least three clear limitations of the present study. First, as this is an uncontrolled study, it is impossible to exclude the possibility that at least some of the observed changes are the result of a vanishing BP effect, rather than a direct SR effect. Second, although this is, to the authors' knowledge, the largest biopsy study in this field of research, the number of patients enrolled might still be too small to explain fully all the effects of SR after BP pre-treatment at the bone tissue level. This study was limited by the number of paired biopsy samples available for analysis and was not powered to detect significant differences in bone quality parameters between treatment groups. Third, as no tetracycline labeling was performed, the bone formation rate is not directly accessible, and any conclusions regarding bone formation are only based on static increases in histomorphometric osteoid and osteoblast indices in conjunction with serum markers of bone formation (increase in bALP) as surrogate parameters.

5. Conclusions

In conclusion, the structural and mineral analyses of iliac crest bone biopsies showed that treatment with SR after long-term treatment with BP leads to significant strontium deposition within the bone, which affects DXA-BMD and qBEI measurements. However, this deposition did not result in mineralization defects or any other detrimental alterations of the mineralized bone matrix. In contrast, a significant increase in osteoid and osteoblast indices and an increase in BV/TV are indicative of newly formed bone in the face of persistent low bone resorption. Therefore, this study suggests that SR might be considered as a therapeutic option in patients, following long-term BP treatment.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1–3, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2010.07.019.

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