



Case Report

Presence of pyrophosphate in bone from an atypical femoral fracture site: A case report



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ABSTRACT

Long-term antiresorptives use has been linked to atypical subtrochanteric and diaphyseal femoral fractures (AFF), the pathogenesis of which is still unknown. In the present case report we present the results of analysis of bone chips from a 74-year old female patient that had been on alendronate, ibandronate and denosumab treatment, and who sustained an atypical femoral fracture, by histology, quantitative backscattered electron imaging, and Raman spectroscopic analysis.

The results indicate ongoing osteoclastic resorption, but also several abnormalities: 1) an altered arrangement of osteons; 2) impaired mineralization; 3) the presence of pyrophosphate, which might contribute to the impaired mineralization evident in the present case. Taken together, these changes may contribute to the focally reduced bone strength of this patient.

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1. Introduction

Suppression of bone turnover has been a major strategic approach in the management of osteoporosis, using therapies such as estrogens, bisphosphonates (BP), or RANK-L inhibitors. Nevertheless, prolonged bone suppression has been hypothesized as the culprit in adverse occurrences such as atypical femoral fractures (AFF) (Shane et al., 2013), although a direct cause-effect relationship has not been established to date. The rarity of such side effects (<1/10,000) suggests, that other mechanisms may play a contributing role (Alonso et al., 2015). To date, altered bone material properties and increased mineralization homogeneity have been hypothesized to be the cause (Ettinger et al., 2013). Recently a case report in JBMR described the occurrence of an AFF in a woman with hypophosphatasia (a condition characterized by accumulation of substrates of alkaline phosphatase such as pyrophosphate, phosphoethanolamine, and pyridoxal 5'phosphate) treated with the bisphosphonates Alendronate and zoledronic acid over a period of 4 years (Sutton et al., 2012).

In the present study we analyzed bone chips obtained from the fracture site during surgery from a 74 year old female patient that had been

on bisphosphonates therapy (mainly alendronate) for more than seven years, followed by treatment with two doses of denosumab, and who sustained an AFF, by histology, quantitative backscattered electron imaging (qBEI) and Raman microspectroscopic (RS) analysis, to explore potential differences in the composition of the AFF bone, including pyrophosphate, as detected by RS (Sutton et al., 2012; Cundy et al., 2015).

2. Materials & methods

The patient was a 74 years-old female, who went through menarche at the age of 14 and menopause at 44 years. She had two children, with accumulated breastfeeding for 17 months. She had no significant clinical problems.

In 2006 (at age 67 yrs) she suffered a fracture of humeral diaphysis. DXA assessment of bone mass revealed a LS-BMD T-score of -4.02 and Femoral Neck T score -2.95. She started weekly alendronate therapy in 2006. Due to upper GI complaints, she changed to monthly ibandronate regimen for a few months in 2008, before returning to weekly alendronate treatment. In December 2012 her GP prescribed denosumab, of which she received two doses. In November 2013, preceded by several weeks of prodromal pain, she suffered a diaphyseal fracture of the right femur due to a fall (five and a half months after the second dose of denosumab). The X-ray revealed a horizontal

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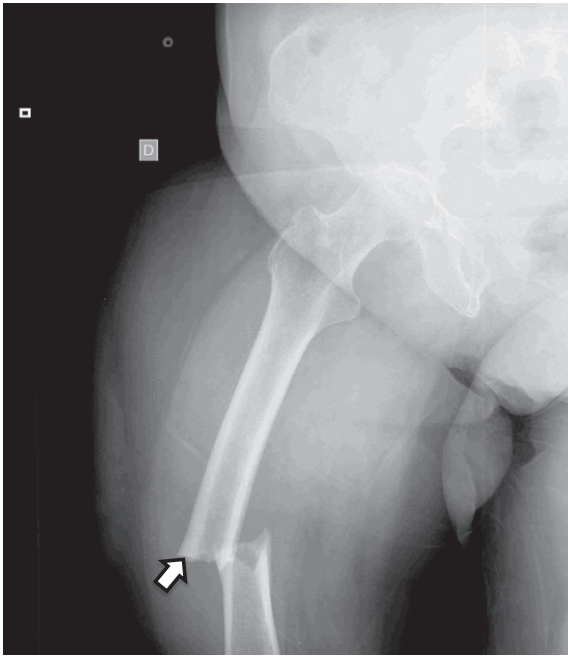


Fig. 1. X-ray of the patient showing the horizontal fracture line and other characteristics of AFF. The area from which the bone chips were collected is indicated by a white arrow.

fracture line and other characteristics of AFF (Fig. 1) as per ASBMR guidelines (Shane et al., 2013). At the time of fracture her lab values were: S-25(OH)D 21.7 (ng/ml); S-PTH 57 pg/ml; S-osteocalcin <2 (below lower limit); CTX 0.15 (0.10–1.00). The fracture was treated within 2 h of occurrence, with a long intramedullary nail and treatment with teriparatide was initiated. Bone chips removed during surgery were subsequently used for the present study (periosteal side of cortical bone). Informed consent was obtained from the patient beforehand. There were no comorbidities when the patient was admitted with the AFF. The only value out of normal range was 25OHvitamin D (21.5 ng/ml). Later on a mild hypercholesterolemia (treated with simvastatin) and mild hypertension (no drug treatment) were detected. The patient, after rehabilitation, is followed up regularly in our outpatient clinic.

2.1. Control bone

As control (CTRL), femoral midshaft bone from an 89 years old female (post-mortem, no sign of any skeletal disease) was used.

2.2. Histology

The bone sample from the AFF site was fixed and dehydrated in ethanol, followed by propanol and xylene, followed by a three-step infiltration with methyl methacrylate. Dehydration and infiltration were performed at 4 °C in a vacuum desiccator and polymerization at –20 °C. The specimen was cut into 7 μm serial sections and dried overnight. Sections were then de-plasticized and stained through the Goldner modification of the Masson trichrome stain.

2.3. qBEI

qBEI analysis was performed on: 1) the bone sample from the AFF site, which was used also for the histological examinations; 2) a transversal 10 mm thick cross section of CTRL bone embedded in PMMA. Instrumental and methodological details have been published elsewhere (Cundy et al., 2015; Roschger et al., 2008; Roschger et al., 1995; Roschger et al., 1998). Five variables were evaluated to characterize the BMDD: Ca_{Mean} , the weighted mean Ca-concentration of the bone area; Ca_{Peak} , the mode of Ca-concentration (the peak position of the histogram); Ca_{Width} , the full width at half maximum of the distribution, describing the variation in mineralization density; Ca_{Low} , the percentage of mineralized bone with a calcium concentration in the bottom 5% of the reference cancellous BMDD (<17.68 wt% Ca); and Ca_{High} the portion of bone areas with a calcium concentration higher than the 95th percentile (>25.30 wt% Ca) of the reference cancellous BMDD (Roschger et al., 2003). In addition to the control bone tissue, the qBEI of BMDD were compared with previously published values from cortical bone of transiliac biopsies samples from postmenopausal patients that were on alendronate or risedronate therapy (CtRef) (Misof et al., 2010).

2.4. Raman spectroscopy (RS)

Raman microspectroscopic analyses were performed on the identical sample blocks (AFF bone sample and CTRL bone sample) used for qBEI analysis after the carbon coating was removed by polishing. Instrumental and methodological details have been published elsewhere (Cundy et al., 2015; Gamsjaeger et al., 2011a; Gamsjaeger et al., 2013; Gamsjaeger et al., 2010; Gamsjaeger et al., 2014a, 2014b; Gamsjäger et al., 2009; Hofstetter et al., 2012). In the bone blocks, 600 individual measurements (each covering an area of $\sim 1 \times 1 \mu m$) were obtained in randomly selected areas of interstitial bone, and the following Raman parameters calculated (Gamsjaeger et al., 2014b; Gamsjäger et al., 2009; Gamsjaeger et al., 2011b; Morris and Mandair, 2011): i) the mineral/matrix ratio (MM), ii) the relative proteoglycan content (PG), iii) the maturity/crystallinity (MMC) of the mineral crystallites, and iv) the relative content of two advanced glycation endproducts (AGEs),

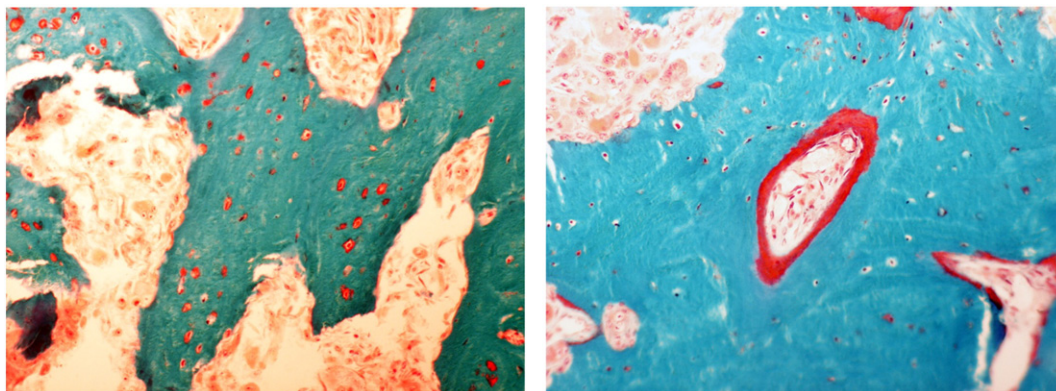


Fig. 2. Light microscopic images of histological sections from bone of the fracture site from AFF patient: Goldner trichrome stained bone sections, left: predominantly primary woven bone with enlarged osteocyte lacunae and numerous resorption lacunae and osteoclasts; right: one osteon like feature with osteoid (red).

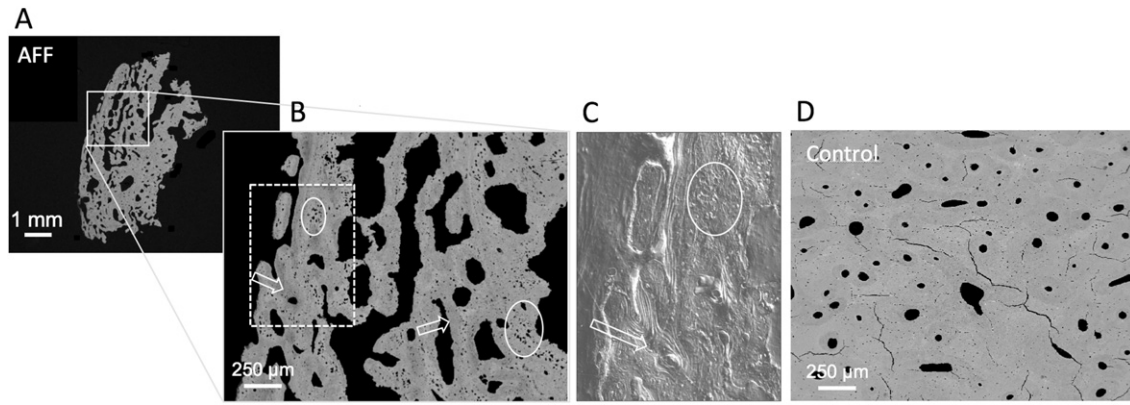


Fig. 3. Section of bone chip at the fracture site of an AFF patient: A) and B) qBEL-image (overview and detail, respectively), circles indicate primary woven bone, arrows lamellar deposited bone. C) Circular polarized light differential interference contrast (C-DIC) image of area in B (indicated by rectangular dashed frame); arrow - lamellar structure clearly visible, circle - enlarged irregular shaped osteocyte lacunae. D) Control compact osteonal bone from femoral midshaft (age, sex matched).

namely CML (ϵ -N-carboxymethyl-L-lysine) and PEN (Pentosidine) (Beattie et al., 2010; Beattie et al., 2011; Glenn et al., 2007; Pawlak et al., 2008). Pyrophosphate (PP) presence and spatial quantification in the bone tissue was determined using the Raman band around 360 cm^{-1} (Chen et al., 2009; Cheng et al., 2009; Fuerst et al., 2010), normalized to mineral content (based on the $\nu_2\text{PO}_4$ band), by RS imaging ($210 \times 130\ \mu\text{m}$ areas) of open osteons.

In addition to the control bone tissue, the Raman results for mineral/matrix, PG, and MMC were compared with previously published values from interstitial bone from postmenopausal patients that were on long term (10 years) ALN therapy (ALN-L), with no AFF incidence (Hassler et al., 2015).

3. Results

Histologic analysis revealed numerous osteoclasts on scalloped bone surfaces. Moreover the remodeling areas were characterized by widened osteoid seams covered by flattened osteoblasts and mineralized bone exhibited enlarged, irregular shaped osteocyte lacunae showing osteocytic osteolysis (Fig. 2).

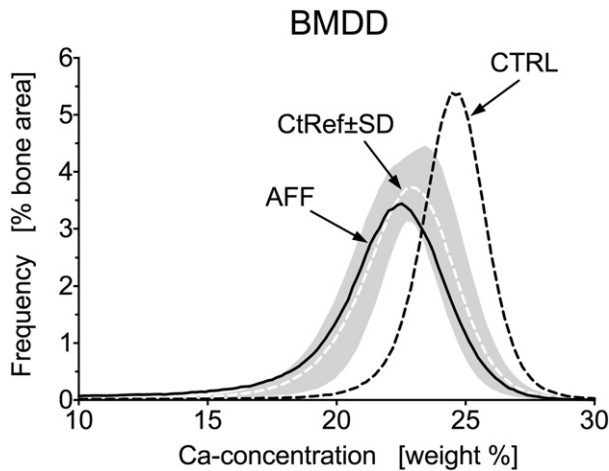


Fig. 4. BMDD characteristics of the bone chips removed from the AFF site indicated that it was shifted towards lower, more heterogeneous mineralization compared to CTRL. The mean and mode calcium content ($\text{Ca}_{\text{Mean}} - 12.3\%$ and $\text{Ca}_{\text{Peak}} - 7.8\%$), were reduced and heterogeneity of mineralization ($\text{Ca}_{\text{Width}} + 46.8\%$), was elevated as well as the percentage of bone with low mineralization ($\text{Ca}_{\text{Low}} + 409\%$) and the percentage of bone with increased mineralization ($\text{Ca}_{\text{High}} - 81\%$) was reduced (Table 1). Moreover the AFF-bone revealed a lower degree of mineralization compared to cortical bone of transiliac bone biopsy samples from postmenopausal osteoporosis patients on alendronate or risendronate therapy (CtRef) (Misof et al., 2010). On the other hand the BMDD of the CTRL-bone was shifted to higher mineralization compared to the CtRef-bone (Table 1).

Backscattered electron imaging of the bone tissue from fracture site of the AFF patient revealed a scaffold of highly mineralized, porous bone matrix with numerous enlarged osteocyte lacunae, on which lamellar bone matrix was laid down (Fig. 3A, B, C) very different from normal compact osteonal bone of CTRL (Fig. 3D). BMDD in AFF-bone was shifted towards lower, more heterogeneous mineralization compared to CTRL. The mean and mode calcium content ($\text{Ca}_{\text{Mean}} - 12.3\%$ and $\text{Ca}_{\text{Peak}} - 7.8\%$), were reduced and heterogeneity of mineralization ($\text{Ca}_{\text{Width}} + 46.8\%$), was elevated as well as the percentage of bone with low mineralization ($\text{Ca}_{\text{Low}} + 409\%$) and the percentage of bone with increased mineralization ($\text{Ca}_{\text{High}} - 81\%$) was reduced (Fig. 4, Table 1). Moreover the AFF-bone revealed a lower degree of mineralization compared to a cortical reference (CtRef) comprised of cortical bone of transiliac crest bone biopsies sample from a cohort of women ($n = 16$) with postmenopausal osteoporosis on either alendronate or risendronate therapy. On the other hand the BMDD of the CTRL-bone was distinctly shifted to higher mineralization compared to the CtRef-bone (Fig. 4, Table 1). RS analysis indicated that the MM of the bone tissue from the AFF patient was lower compared to CTRL (Fig. 5a), while the PG content and MMC values were higher in the AFF case (Fig. 5b and c, respectively). Both relative CML and PEN contents were elevated in the AFF case compared to CTRL (Fig. 5d and e, respectively). RS analysis for the presence of pyrophosphate, revealed its presence above instrument detection limit only in the AFF case (Fig. 6). No significant differences were evident between the AFF and the ALN-L in the mineral/matrix ratio and MMC values, unlike the PG where the AFF had elevated ones (Fig. 5a–c).

4. Discussion

AFFs, although rare, have been identified as severe complications of prolonged bone turnover suppression in osteoporotic patients on antiresorptive therapies. No mechanism has been conclusively proven yet for their occurrence, though alterations in the bone material properties have been proposed as a culprit. In the present study we analyzed bone chips removed during surgery at the fracture site from a 74-yr old patient that had sustained an AFF and who had been on ALN therapy

Table 1
Corresponding values of BMDD parameters.

BMDD-parameter	CtRef	CTRL	AFF	Diff%
Ca_{Mean} [wt% Ca]	22.26 ± 0.7172	24.19	21.22	-12.3
Ca_{Peak} [wt% Ca]	22.81 ± 0.7288	24.43	22.53	-7.8
Ca_{Width} [wt% Ca]	3.710 ± 0.3136	2.95	4.33	+46.8
Ca_{Low} [%]	4.317 ± 1.452	2.05	10.45	+409.8
Ca_{High} [%]	-	28.98	5.50	-81.0

CtRef = mean cortical BMDD parameters (mean \pm SD, $n = 16$) from published values (Misof et al., 2010); Diff% = percentage of difference between CTRL and AFF.

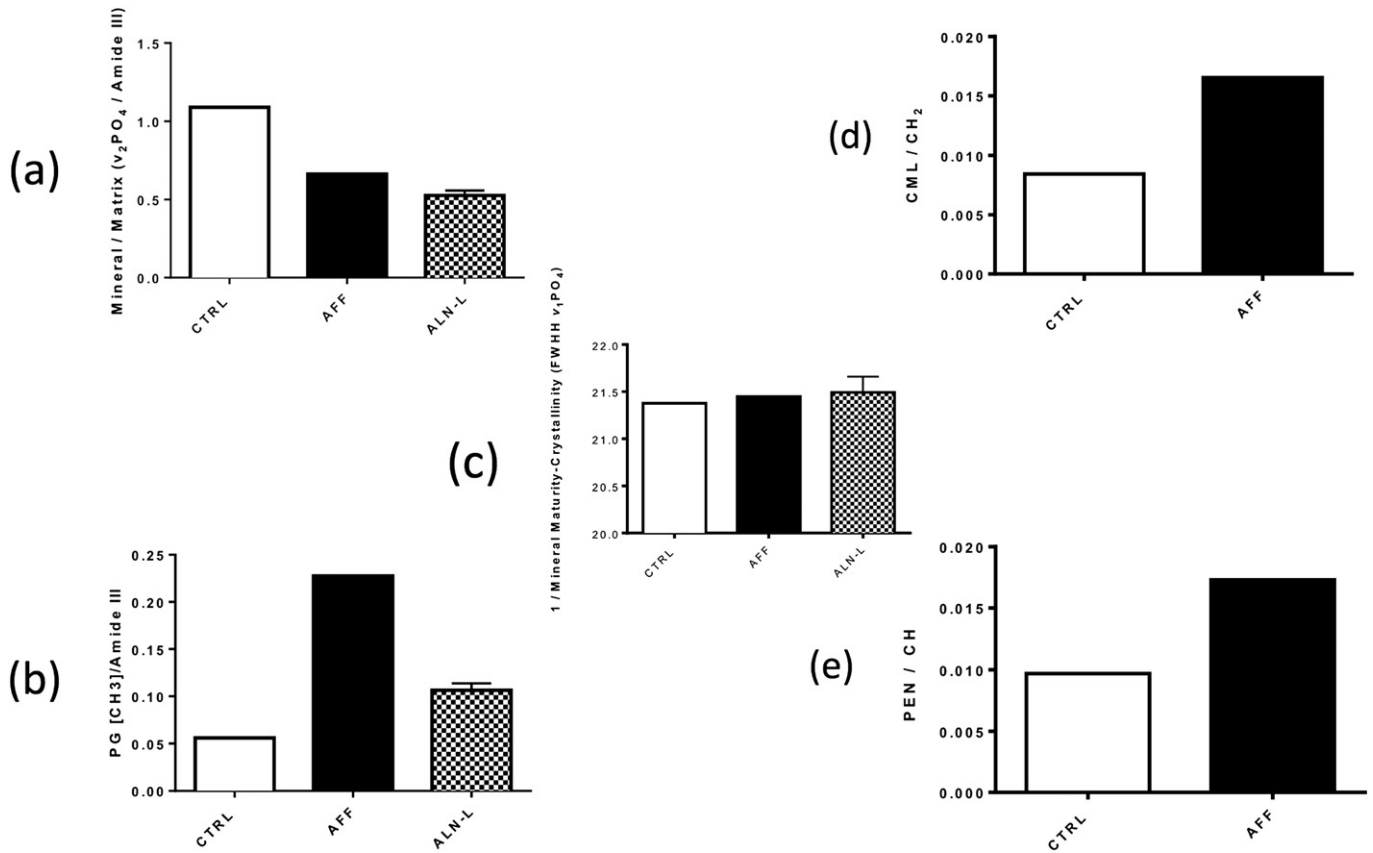


Fig. 5. Raman spectroscopic analysis outcomes: mineral/matrix (a), relative proteoglycan content (b), mineral maturity/crystallinity (c), CML (d), and pentosidine (e). The bone chip from the AFF-sustaining patient (AFF) was compared against bone a site-, age-, and sex-matched bone from a healthy donor (CTRL), and postmenopausal osteoporosis patients treated with long-term (10 years) alendronate therapy (ALN-L).

for 6 years, interrupted by a brief period on ibandronate and followed by a brief administration of denosumab. Distinct changes at the bone tissue and material level were observed at this skeletal site, when compared with site, age and sex matched healthy control bone.

4.1. Tissue level

The femoral bone chips examined in the present study exhibited an abnormally high porosity and distinct deviations from a compact

osteonal bone structure as found in normal femoral diaphysis, consistent with previously published observations based on X-rays (Shane et al., 2013; Shane et al., 2010), and scintigraphy (Shane et al., 2010) considerations. qBEI and C-DIC images (circular polarized light differential interference contrast imaging) revealed a scaffold of highly mineralized, porous bone matrix with enlarged osteocyte lacunae, on which lamellar bone was laid down. The entire tissue was very different from normal compact osteonal bone of CTRL. Histological analysis revealed numerous osteoclasts on scalloped bone surfaces, widened osteoid

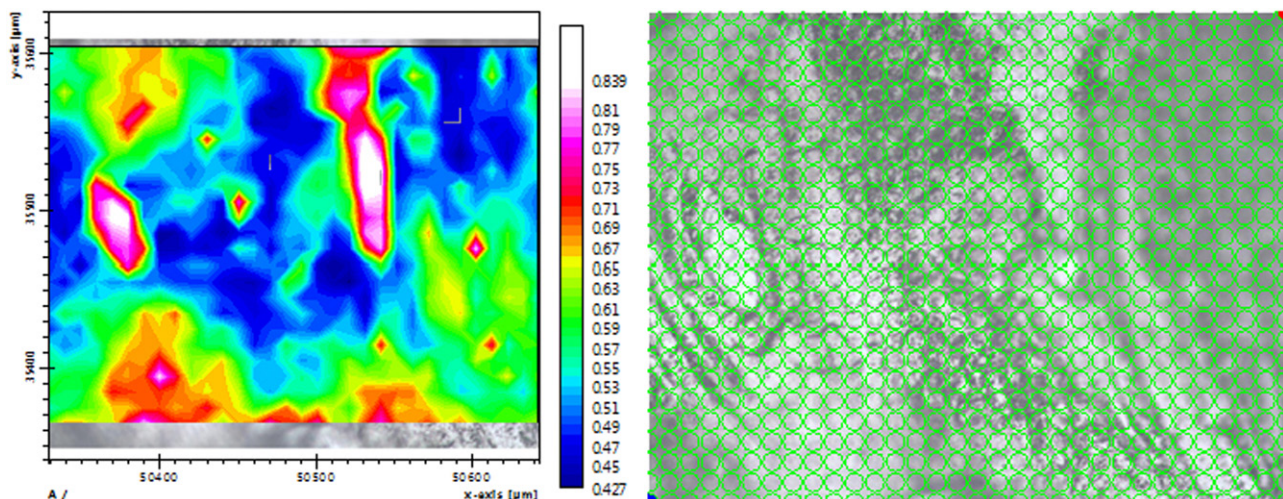


Fig. 6. Results of the Raman imaging analysis of the bone chip from the patient with the atypical fracture. A photomicrograph of the imaged area is shown on the right, while the calculated Raman image based on the height of the typical band for pyrophosphate (PP) -360 cm^{-1} (normalized to mineral content) is shown on the left.

seams and enlarged irregular shaped osteocyte lacunae, while hardly any osteoblasts were observed, suggesting that at the fracture site, bone was continuously transformed from normal osteonal compact bone to a highly porous one with disturbed osteonal arrangement, potentially as a response to focally ongoing fatigue damage processes.

4.2. Material level

Compared to the CTRL-bone sample the AFF-bone showed a general shift to lower matrix mineralization. Three scenarios can be responsible for this: 1) the average tissue age of the AFF bone is significantly younger than the CTRL due to an increased bone turnover rate; 2) the bone matrix mineralization kinetics are altered; 3) a combination of 1 and 2. The lack of any osteoblastic activity in the AFF-bone suggests that the second hypothesis is more plausible. It is also supported by the presence of pyrophosphate (mineralization inhibitor), which was detected only in AFF. The strongly increased width of the BMDD-peak is likely reflecting that two bone types are present in AFF-bone: the primary woven bone with a relative high matrix mineralization and the lamellar bone of lower mineral content. It is also noteworthy that the osteonal CTRL-bone is distinctly higher mineralized than cancellous bone of a normative reference data base (Roschger et al., 2003) most likely indicating differences in average matrix mineralization density between these skeletal sites (Fratzl-Zelman et al., 2009).

In contrast to qBEI, RS analysis was performed on single points with focus directed towards the oldest tissue in the bone sample (interstitial bone areas), with exception of the pyrophosphate measurements where open osteons were also considered.

The MM of the AFF-bone was lower than CTRL-bone, in agreement with the qBEI results. It was also similar to the ALN-L patients, suggesting that the present AFF may not be necessarily attributed to the long term antiresorptive therapy as far as this bone quality index is concerned. PG (negative modulators of mineralization (Boskey et al., 1997; Mochida et al., 2003; Mochida et al., 2009; Nielsen et al., 2003; Bi et al., 2006; Xu et al., 1998; Sauren et al., 1992; Thompson et al., 2011; Gualeni et al., 2013; Ohtsuki et al., 1995)) content of the AFF case was higher compared to either CTRL or ALN-L, possibly contributing to the lower mineralization measured in the AFF case. The MMC (Fratzl et al., 2004; Donnelly et al., 2012a) of the AFF case was similar to the CTRL and ALN-L, suggesting that it may not be contributing to the AFF occurrence, and also argues against the contribution of active fracture repair mechanisms (Ouyang et al., 2004) as would be expected given that the patient was operated within 2 h of fracture occurrence.

On the other hand, the relative CML and PEN content was elevated in the AFF case compared to CTRL, potentially contributing to the fragility fracture as AGEs accumulation is believed to be detrimental to bone strength (Karim et al., 2013).

A recent report described the development of an AFF in a patient with hypophosphatasia being treated with zoledronic acid (Sutton et al., 2012). Inorganic pyrophosphate is a primary antagonistic regulator of extracellular matrix mineralization (Zhou et al., 2012), through its competition against inorganic phosphate in the promotion of hydroxyapatite deposition (Moochhala et al., 2008; Harmey et al., 2004; Clarke, 2008; Anderson, 2003). In the present study we explored for its presence in areas of open osteons, by RS imaging analysis. The only specimen that exhibited detectable pyrophosphate levels by RS was the AFF one. The lack of PP detection in all other groups may be due to the fact that in healthy bone, PP levels are well below 1% (Perkins and Walker, 1958).

A recent publication, reported on the cortical and trabecular bone tissue properties and compositional heterogeneity near fragility fracture sites of patients that were receiving either BP or another osteoporosis therapy. Among the BP-receiving patients sustaining fragility fractures, 6 had AFF, 5 of which were on ALN and one on ibandronate therapy (Donnelly et al., 2012b). The values for MM in the AFF subgroup were no different compared to the non-AFF fragility fracture sub-group,

unlike the present results. Moreover, the mineralization heterogeneity was significantly reduced in the AFF sub-group, unlike the present results. However, MMC in the AFF subgroup was no different compared to the non-AFF fragility fracture sub-group, in agreement with the results of the present study. A possible reason for this discrepancy could be that in the previous study (Donnelly et al., 2012b), bone close to the fracture site was analyzed, while in the present one a bone chip from the actual fracture site was utilized, and as per ASBMR definition, AFF is a focal weakness in bone thus analyzing bone from neighboring sites may not be completely representative of the material properties of an AFF site. Also in the already published study (Donnelly et al., 2012b), all the analyzed patients were on antiresorptive therapy, whereas in the present case the control tissue was from a necropsy specimen not treated with any antiresorptives. On the other hand, it should be kept in mind that the mineral/matrix and MMC values as determined by RS were not different from the ones calculated in bone tissue from patients on long-term ALN therapy and who did not suffer any AFF, suggesting that antiresorptive therapy may not be reason for the described discrepancies.

A unique finding of the present study is the presence of PP in the examined tissue at the fracture site of the AFF, which coupled to the elevated PG values may be contributing to the lower mineral content and delayed mineralization of bone matrix.

A limitation of the present study is that the control tissue we used was from a necropsy specimen that was 15 years older than the AFF patient, and was deemed as healthy bone, as we do not have access to bone tissue from a better age-matched, treatment-naïve postmenopausal osteoporosis patient without any AFF incidence. On the other hand, for the RS analyses, we compare the properties of the present AFF bone tissue with those obtained in postmenopausal osteoporosis patients who had been treated long term with antiresorptive therapy without sustaining AFF. Moreover, for the qBEI analysis, we compared the bone matrix mineralization of AFF bone with cortical bone of transiliac bone biopsy samples from postmenopausal osteoporosis patients on alendronate or risendronate therapy.

In conclusion, bone chips removed from the fracture site of an AFF case showed ongoing osteoclastic resorption despite antiresorptive treatment, an impaired arrangement of osteons, increased heterogeneity in mineralization, elevated proteoglycan, CML, and PEN content, and PP accumulation. Taken together, these changes may contribute to the focally reduced bone strength at the site of AFF in the present case. The observations made are compatible with the assumption of an ongoing history of focal fatigue damage coupled to a form of repair response at the fracture site.

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All authors made substantial contributions to either the conception and design, acquisition of data or analysis and interpretation of data, participated in drafting the manuscript or revising it critically for important intellectual content, and approved the final version of the submitted manuscript.

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