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Case Report

Organic matrix quality discriminates between age- and BMD-matched fracturing versus non-fracturing post-menopausal women: A pilot study

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ABSTRACT

Women with similar areal Bone Mineral Densities (BMD) may show divergent fracture incidence due to differences in bone quality.

The hypothesis tested in the present pilot study is that postmenopausal (PM) women who have sustained osteoporotic fractures have altered organic matrix quality compared to those who have not. We used Raman microspectroscopy to analyze transiliac biopsies collected from fracturing (n = 6, mean age 62.5 ± 7.4 yrs; Cases) and non-fracturing PM women (n = 6, age- and BMD-matched; mean age 62.2 ± 7.3 yrs; Controls). Previous results show differences in intrinsic material properties by nanoindentation that are more homogenously distributed and could facilitate microcrack propagation in Cases, along with lower mineral carbonate/phosphate ratio by Fourier transform infrared spectroscopic imaging, and no differences in bone tissue mineralization by digitized microradiography. No differences between groups were seen by conventional histomorphometry.

Spectra were acquired 2 μm away from previously performed nanoindents, in cortical and cancellous compartments. The determined parameters were: mineral to matrix ratio (MM), and nanoporosity (a surrogate for tissue water (TW)), glycosaminoglycan (GAG), pyridinoline (Pyd; trivalent enzymatic collagen cross-link), N(6)-carboxymethyllysine (CML; advanced glycation endproduct), and pentosidine (PEN; advanced glycation endproduct) content.

ANCOVA indicated no differences in any of the spectroscopic outcomes between cancellous and cortical compartments. On the other hand, Cases had lower nanoporosity (TW) and GAG, and elevated Pyd, and CML content compared to Controls.

In conclusion, the results of the present study indicate significant differences in organic matrix quality in PM women that sustain fragility fractures versus age- and BMD-matched controls, highlighting its importance as a potential independent determinant of fracture incidence.

1. Introduction

Loss of bone mass, measured clinically as change in bone mineral density (BMD), is considered an important risk factor for bone fragility. However, BMD is not the sole predictor of whether an individual will experience a fracture [1,2], and there is considerable overlap in BMD between populations that do and do not develop fractures [3–5]. It has been demonstrated that for a given bone mass an individual's risk to fracture increases with age [6]. Consistent with these findings, numerous investigators have shown that mechanical variables directly related to fracture risk are either independent [7] or not totally accounted for bone mass itself [8–12]. Epidemiological evidence also shows considerable overlap of bone density values between fracture and non-fracture groups suggesting that low bone quantity alone is an insufficient cause of fragility fractures [13–18]. In fact, a recent article [19] determined that that BMD by DXA is not a reliable worldwide screening tool to predict fractures even when implemented with assessment tools such as FRAX®. This is supported by an even more recent study comparing the US and Canadian osteoporosis screening and treatment strategies in postmenopausal women [20]. It has become

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well accepted then, that in addition to BMD, other factors should be considered when assessing bone strength and fracture risk.

A recent study tested the hypothesis that postmenopausal women who have sustained osteoporotic fractures have reduced bone quality, as indicated with measures of intrinsic material properties compared to those who have not fractured [21]. Iliac crest biopsies collected from postmenopausal women without any fragility fractures (Controls), and age- and BMD-matched controls, were analyzed by nanoindentation. The results indicated that Cases had significantly lower median values for cortical hardness and indentation modulus, and exhibited significantly less within-specimen variability in cortical modulus, cortical hardness, cortical storage modulus and trabecular hardness, and more within-specimen variability in trabecular loss modulus, suggesting that mechanical heterogeneity of bone tissue is an important contributor in the determination of bone strength [21]. Conventional histomorphometry did not show any significant differences between the two groups [21]. Fourier transform infrared imaging (FTIRI) of randomly selected areas showed that Cases had significantly lower carbonate/phosphate ratio [22]. Analysis of the whole biopsy surface by digitized microradiography (DM) did not indicate any significant differences between Controls and Cases in either degree of mineralization (DMB) or the heterogeneity index (HI) [23].

In the present pilot study, we used Raman microspectroscopy to analyze a randomly selected sub-set of epoxy resin embedded transiliac biopsies (N = 12) collected from fracturing (n = 6, mean age 62.15 ± 4.4 yrs; Cases) and non-fracturing PM women (n = 6, age- and BMD-matched; mean age 61.54 ± 4.0 yrs; Controls). We took advantage of the spatial resolution of this technique (~1 μm) to analyze the immediate area surrounding the previously performed nano-indents [21], to test the hypothesis that the organic matrix quality is different between Controls and Cases. The spectroscopically determined parameters were the mineral to matrix ratio as well as tissue water, glycosaminoglycan, lipid, pyridinoline, N(6)-carboxymethyllysine (CML), and pentosidine (PEN) contents.

2. Materials & methods

2.1. Patients

Details for the complete cohort of patients have been published elsewhere [21]. The iliac crest biopsies analyzed in the present pilot study were provided by Dr. Recker from his Bone Quality Study, with consent of the "Bone Quality Analysis Study Team". Written consent was obtained from all participants. The study was performed under IRB 07-14738 from Creighton University and EK 17-096-VK from the Ludwig Boltzmann Institute for Osteology. In the initial study, biopsies from women with fractures were paired with non-fracture ones by matching the non-fracture subjects to within 5 years of the age, and within 10% of the hip BMD of each fracture case [21,22]. Nevertheless, the age matching criteria are arbitrary ones and may not be robust [20], thus in the present study we treated individual patient age as a covariant.

The fracture group (Cases) had osteopenic BMD values (T-scores between −1.0 and −2.5 for either the hip or spine), had a fracture during the previous 5 years from low trauma (defined as any fracture caused by trauma equal to, or less than, a fall to the floor from a standing height, excluding fractures of the digits, face or skull), but were otherwise healthy [21]. None of the women in either group were on any anti-resorptive (bisphosphonate, calcitonin, estrogen, etc.) or bone forming (PTH) agents [21]. The mean age for the sub-set analyzed in the present study was 61.54 for the Control (minimum 57.61, maximum 68.08 years), and 62.15 (minimum 56.62, maximum 67.3) years for the Case patients. Pre-study clinical evaluation did not reveal any findings of "flat feet", scoliosis, or any "looseness" present in either Cases or Controls.

2.2. Raman analysis

Raman microspectroscopic analyses (with a spatial resolution of ~1 μm) employed a Senterra (Bruker Optik GmbH) instrument. A continuous laser beam was focused onto the sample through a Raman fluorescence microscope (Olympus BX51, objective 50×) with an excitation of 785 nm (100 mW) and a lateral resolution of ~0.6 μm. The Raman spectra were acquired from the bone surface, using a thermoelectric–cooled charge-coupled device (CCD) (Bruker Optik GmbH). All data analysis was done with the Opus Ident software package (OPUS 6.5, Bruker Optik GmbH). Once acquired, the Raman spectra were baseline corrected (rubber band, 5 iterations) to account for fluorescence, and the following parameters were calculated:

(i) the mineral/matrix ratio from the integrated areas of the ν_2PO_4 (410–460 cm^{-1}) to the amide III (1215–1300 cm^{-1}) bands [24], which has been shown to be independent of tissue organization [24]
(ii) Nanoporosity (tissue water) was approximated by the ratio of the integrated areas of the spectral slice 494–509 cm^{-1} (PMMA) to Amide III band [25,26]. This metric is a surrogate of tissue water content at the sub-micron level.
(iii) the relative proteoglycan content was expressed as the proteoglycan/matrix ratio (the ratio of the integrated areas of the proteoglycan/CH_3 [1365–1390 cm^{-1}] band [representative of mucopolysaccharides] [27–29] to the Amide III [1215–1300 cm^{-1}] band) [30]
(iv) The relative content of two AGEs, namely CML (ε-*N*-carboxymethyl-L-lysine) and PEN (pentosidine) was monitored as the integrated area ratio of bands at 1150 (CML) cm^{-1} or 1495 (PEN) cm^{-1}/1450 cm^{-1} (methylene side chains (CH_2)) [31–36]
(v) the relative PYD content was calculated as the absorbance height at 1660 cm^{-1}/area of the amide I (1620–1700 cm^{-1}) [37–40]
(vi) the maturity/crystallinity (MMC) of the bone mineral apatite crystallites was approximated from the inverse of the full width at half height (FWHH) of the ν_1PO_4 (930–980 cm^{-1}) band, which correlates with crystallite length (002 crystallographic reflection) [41].

2.3. Anatomical area selection criteria

Spectra were acquired 2 μm away from previously performed nanoindents [21], in cortical and cancellous compartments. Twenty such anatomical areas per biopsy per compartment were analyzed, the results averaged and the mean value treated as a single statistical unit for the biopsy compartment.

2.4. Statistical analysis

Data were subjected to analysis of covariance with weighted least squares regression (WLS; for each parameter, the standard deviation of all twenty measurements in each patient analyzed was used as WLS weight), with the two groups (Controls vs. Cases) and the two anatomical locations (cortical vs. trabecular) as fixed factors, and individual patient age as a covariate, followed by Sidak's post-hoc tests. In all instances, statistical significance was assigned to $p < 0.05$.

3. Results

The mean and standard deviation values for the nanoindentation and spectroscopic parameters determined in the patient group considered in the present study are listed in Table 1 as a function of anatomical compartment and fragility fracture incidence.

Fig. 1 shows typical photographs obtained through the Raman microscope in the cortical (left) and cancellous (right; obtained with fluorescence source on so that adjacent double labels denoting active

Table 1
Summary of the mean values and corresponding standard deviations for the nanoindentation and spectroscopic analysis of the patients considered in the present study, as a function of anatomical compartment and fragility fracture incidence. For each nanoindentation outcome, the variability of the standard deviations is also provided as a surrogate for heterogeneity.

	Controls				Cases			
	Cortical		Cancellous		Cortical		Cancellous	
	Mean	Std. deviation	Mean	Std. deviation	Mean	Std. deviation	Mean	Std. deviation
Hardness (GPa)	1.025	0.44599	0.3867	0.07367	0.3367	0.17795	0.3	0.04858
Standard deviation hardness	0.2133	0.11057	0.0667	0.03141	0.07	0.05215	0.0517	0.01472
Modulus	20.5633	4.43481	14.595	2.97429	10.6433	3.52322	12.4767	2.37895
Standard deviation modulus	2.84	1.28206	1.9233	0.82439	1.44	0.81677	1.6433	0.38077
Storage modulus	20.4083	2.94227	16.82	2.85026	12.4933	2.99768	15.7067	2.79451
Standard deviation storage modulus	2.7333	1.1013	1.6867	0.67491	1.7515	0.98848	1.7983	0.36679
Loss modulus	0.7167	0.15782	0.5583	0.18883	0.59	0.38941	0.6817	0.27309
Standard deviation loss modulus	0.1683	0.09725	0.1	0.0645	0.145	0.0745	0.195	0.12755
Tan delta	0.0357	0.00981	0.0333	0.00894	0.0473	0.02886	0.0438	0.01768
MM	0.4869	0.16537	0.5104	0.29535	0.5135	0.17482	0.4095	0.12954
NanoP	0.2393	0.06156	0.2665	0.12694	0.0931	0.0627	0.0918	0.08456
GAGs	0.0543	0.01605	0.0514	0.01487	0.0403	0.01137	0.0402	0.00804
Pyd	0.011	0.00485	0.0113	0.00259	0.0159	0.00142	0.0155	0.00157
MMC	0.0484	0.00146	0.0489	0.00104	0.0485	0.00162	0.0485	0.00128
CML	0.14	0.10036	0.1615	0.09732	0.485	0.3586	0.3462	0.2281
PEN	0.0373	0.02546	0.0395	0.02591	0.0497	0.02497	0.0628	0.01911

bone formation are visible) compartments, demonstrating the points selected for Raman analysis (red crosses).

ANCOVA results for the nanoindentation tests outcomes for the 12 patients employed in the present pilot study are shown in Table 2 and Fig. 2 (only significantly different parameters between Controls and Cases are shown). Hardness and hardness standard deviation (hardness SD; a surrogate for heterogeneity distribution) were significantly higher in the cortical compared to trabecular bone. Controls had higher hardness, modulus and storage modulus values compared to Cases, as well as increased heterogeneous distribution for the hardness and modulus outcomes compared to Cases (Table 2), in agreement with previously published results on the whole cohort [21].

ANCOVA results for the spectroscopic outcomes for the 12 patients employed in the present pilot study are shown in Table 3 and Fig. 3 (only significantly different parameters between Controls and Cases are shown). Unlike the nanoindentation outcomes, there were no significant differences between cortical and trabecular compartments. On the other hand, Controls had significantly higher tissue water, and GAG content, and significantly lower Pyd and CML content compared to Cases.

4. Discussion

Despite BMD being the mainstay of the clinical diagnostic arsenal, it is well recognized that the mechanical properties of bone tissue depend both on the mineral and the matrix (primarily type I collagen fibrils) constituents [42–47]. Thus, it is not surprising that bone density measurements account for ~50% of the variation in risk of fracture [48–51].

Previous studies comparing bone properties in a cohort of age- and BMD-matched postmenopausal osteoporosis patients some of whom sustained osteoporotic fractures (Cases) while others did not (Controls), revealed significant differences in intrinsic material properties by nanoindentation. These properties were more homogenously distributed and could propagate microcracks more easily in Cases. Additionally, Cases had lower mineral carbonate/phosphate ratio by Fourier transform infrared spectroscopic imaging (FTIRI). No differences between the two groups in bone tissue mineralization were observed by digitized microradiography. Finally, no differences between groups were seen by conventional histomorphometry [21–23]. The lack of major differences between the two sub-groups, along with previously published studies showing that in both animal models and humans organic matrix

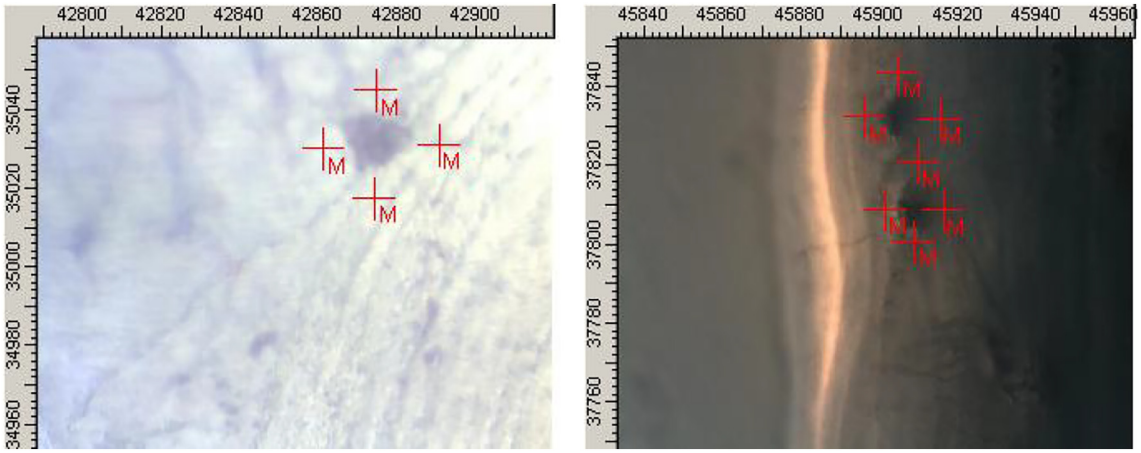

Fig. 1. Typical photographs taken through the Raman microscope in the cortical (left) and cancellous (right; picture was acquired with the fluorescent source on to show the presence of a double label in the vicinity of the nanoindents) compartments, showing the areas of Raman analysis (red crosses). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
ANCOVA results of the nanoindentation outcomes for the biopsies analyzed in the present pilot study. Statistical significance is denoted by bold typeface.

Pairwise comparisons

Dependent variable	(I) Compartment	(J) Compartment	Mean difference (I − J)	*p*-Value
Hardness	**Cortical**	**Trabecular**	**0.459***	**0.026**
Hardness SD	**Cortical**	**Trabecular**	**0.083***	**0.005**
Modulus	Cortical	Trabecular	2.715	0.100
Modulus SD	Cortical	Trabecular	0.357	0.309
Storage modulus	Cortical	Trabecular	0.616	0.623
Storage modulus SD	Cortical	Trabecular	0.5	0.15
Loss modulus	Cortical	Trabecular	0.076	0.526
Loss modulus SD	Cortical	Trabecular	0.009	0.814
Tan delta	Cortical	Trabecular	0.003	0.702

Pairwise comparisons

Dependent variable	(I) Health	(J) Health	Mean difference (I − J)	*p*-Value
Hardness	**Controls**	**Cases**	**0.436***	**0.034**
Hardness SD	**Controls**	**Cases**	**0.077***	**0.008**
Modulus	**Controls**	**Cases**	**5.722***	**0.002**
Modulus SD	**Controls**	**Cases**	**0.790***	**0.033**
Storage modulus	**Controls**	**Cases**	**4.598***	**0.001**
Storage modulus SD	Controls	Cases	0.399	0.248
Loss modulus	Controls	Cases	−0.103	0.403
Loss modulus SD	Controls	Cases	−0.033	0.405
Tan delta	Controls	Cases	−0.011	0.174

properties strongly associate with fragility fracture incidence independent of estimated fracture risk [16–18,52–54] led to the present pilot study. In this study, we investigated tissue properties around previously performed nano-indents [21] to test the hypothesis that bone organic matrix quality is different between Control and Case patients.

The mechanical outcomes in the present randomly selected sub-set of biopsies were in general agreement with what has been reported for the whole cohort [21], supporting the assumption that the sub-set analyzed in the present study may be representative of the whole cohort. Hardness and hardness heterogeneity (approximated by the standard deviation) were greater in the cortical compared to trabecular compartment, and were also greater in the Controls compared to Cases. Moreover, Controls also had higher modulus, modulus heterogeneity,

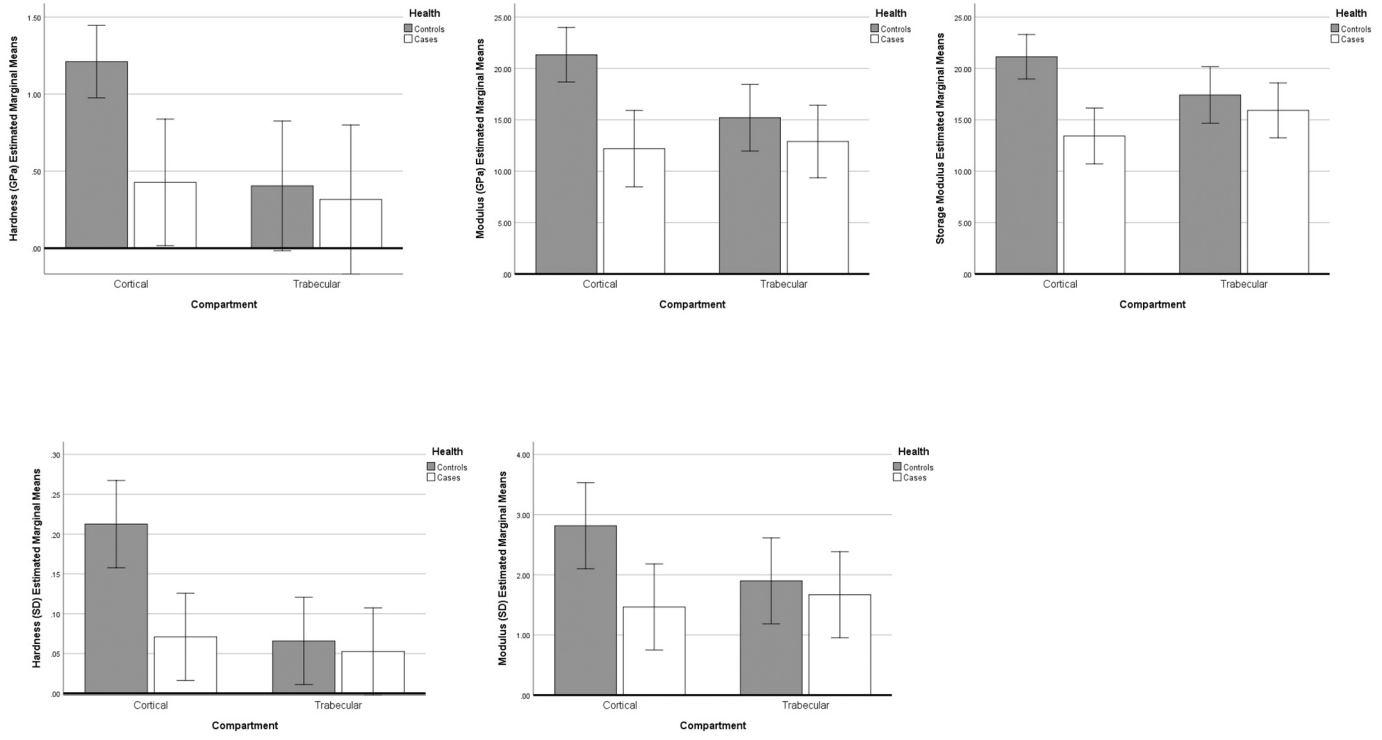

Fig. 2. ANCOVA analysis of the nanoindentation outcomes for the biopsies analyzed in the present study indicated that Hardness and hardness heterogeneity (approximated through the standard deviation) were higher in the cortical compartment compared to trabecular, and were also significantly higher in the Controls (grey columns) compared to Cases (open columns) (Table 2). Controls also had significantly higher modulus and storage modulus as well as modulus heterogeneity values compared to Cases. In all plots the estimated marginal means and the 95% confidence interval plotted as error bars are shown.

Table 3
ANCOVA results of the spectroscopic outcomes for the biopsies analyzed in the present pilot study. Statistical significance is denoted by bold typeface.

Pairwise comparisons

Dependent variable	(I) Compartment	(J) Compartment	Mean difference (I − J)	*p*-Value
Mineral/matrix	Cortical	Trabecular	−0.29	0.735
Nanoporosity (tissue water)	Cortical	Trabecular	0.00021	0.995
Glycosaminoglycans	Cortical	Trabecular	0.001	0.842
Pyridinoline	Cortical	Trabecular	−0.001	0.436
Mineral maturity/crystallinity	Cortical	Trabecular	−0.000485	0.375
CML	Cortical	Trabecular	0.04	0.652
PEN	Cortical	Trabecular	−0.000008	0.999

Pairwise comparisons

Dependent variable	(I) Health	(J) Health	Mean difference (I − J)	*p*-Value
Mineral/matrix	Controls	Cases	0.085	0.211
Nanoporosity (tissue water)	**Controls**	**Cases**	**0.176***	**< 0.0001**
Glycosaminoglycans	**Controls**	**Cases**	**0.015***	**0.013**
Pyridinoline	**Controls**	**Cases**	**−0.004***	**< 0.0001**
Mineral maturity/crystallinity	Controls	Cases	0.00048	0.385
CML	**Controls**	**Cases**	**−0.195***	**0.037**
PEN	Controls	Cases	−0.017	0.051

and storage modulus values than Cases.

Our spectroscopic analysis focusing on the tissue properties in the immediate vicinity of the nanoindents, indicated no significant differences between cortical and trabecular compartments. No differences in the mineral/matrix ratio or the mineral maturity/crystallinity between Controls and Cases were evident either, consistent with the previously lack of any differences in either by FTIRI and DM analyses [22,23]. These results suggest that the observed differences in the intrinsic mechanical properties of the two groups may not be attributed to either the quantity or the quality of the bone mineral.

On the other hand, Cases had significantly different organic matrix properties compared to Controls. They had lower tissue water and GAG contents. Tissue water, at all hierarchical levels, strongly associates with mechanical properties [55,56], as it contributes to the overall toughness of the bone composite, serving as a plasticizer [57]. It is therefore entirely plausible that this decrease in tissue water content in the Case patients contributes to the bone fragility evident in them. Cases also had significantly lower GAG content, a finding consistent

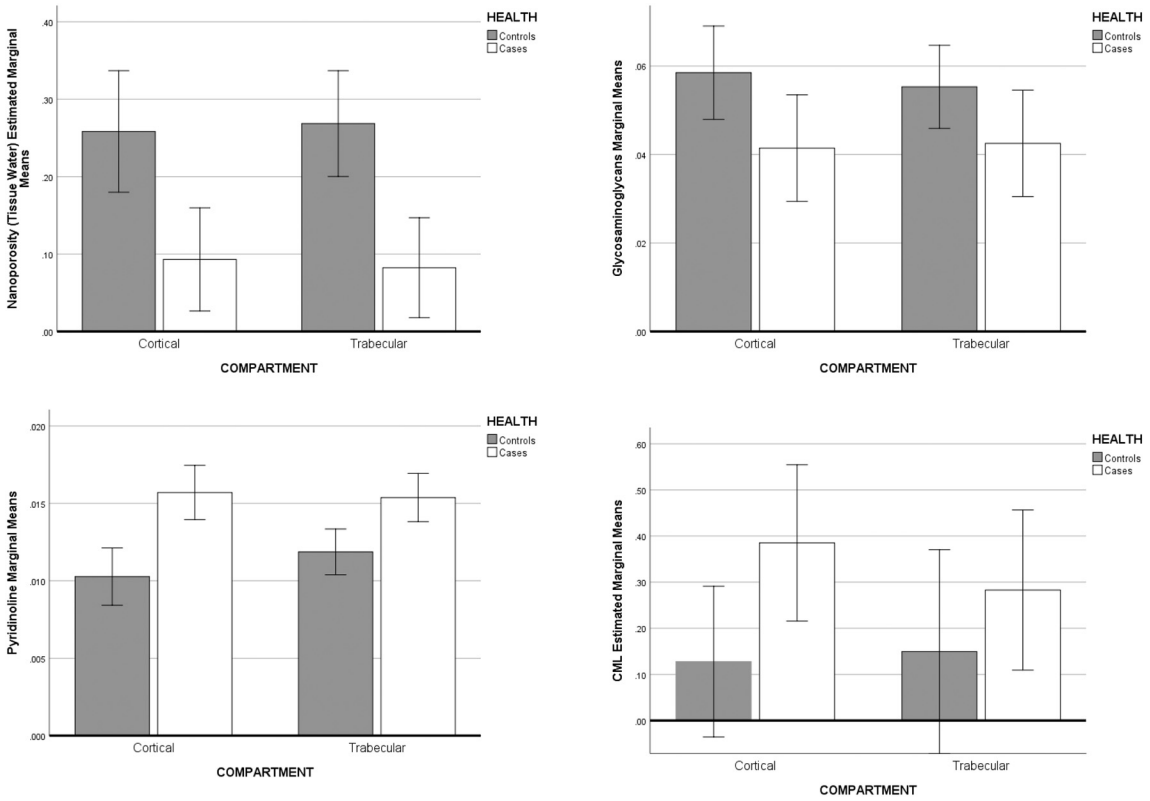

Fig. 3. ANCOVA analysis of the spectroscopic outcomes revealed no differences between Controls (grey columns) and Cases (open columns) in either the mineral/matrix ratio or the mineral maturity/crystallinity. On the other hand, Cases had significantly lower nanoporosity and GAG content, and elevated Pyd and CML content compared to Controls (Table 3). In all plots the estimated marginal means and the 95% confidence interval plotted as error bars are shown.

with the decrease in tissue water content, as they have been shown to act like "sponges" adsorbing water molecules [58]. The micro-anatomical areas analyzed in the present study were in interstitial bone, away from forming or resorbing surfaces. As such, one may hypothesize that the major source of the GAGs measured in the present study are due to the proteoglycans (PG) present in the canalicular network. In vitro, in situ, and in vivo experiments have established PGs as negative modulators of mineralization [59–61], and have also indicated their presence in perilacunar matrix around the osteocyte lacunae, and around the canaliculi [62] in compact lamellar rat and human bone, leading to the proposal that a potential role of these osteocyte-related proteoglycans (and in particular perlecan/*Hspg2* (PLN)) is to keep the pericellular space of the lacunocanalicular network mineral-free so as to ensure uninhibited interstitial fluid movement [63]. Thus, it is plausible that this significant decrease in GAG content in Cases is in part due to altered canalicular network, in general agreement with previously published data showing changes in canalicular network as a function of aging and disease [64–70]. It has been previously shown that collagen orientation at the equatorial perilacunar region in combination with the presence of the lacuna itself affect the mechanical competence of bone [71]. In the present work, we avoided analyzing osteocyte lacunae. Additionally, the Raman peak used for the determination of the organic matrix (Amide III) has been shown to be tissue organization/orientation independent [24,30,72,73], thus it is not feasible to compare the results of the present study with the previously published ones [71].

Cases had significantly higher Pyd content compared to Controls. Pyridinoline is a mature, enzymatic, non-reducible, trivalent collagen cross-link abundant in mineralized tissues [74]. Collagen cross-linking chemistry and molecular packing structure is a hallmark of type I collagen in mineralizing tissues [74], responsible for fibrillar matrices mechanical properties such as tensile strength and viscoelasticity, thus are important determinants of bone strength [53,75–77]. Changes in enzymatic cross-links, even when confined to micro-anatomical locations, have been shown to be capable of affecting the mechanical attributes of the whole bone organ [53,77], and have consistently and strongly associated with fracture incidence rather than fracture risk in instances the two are divergent [16–18,52,78]. Collagen fibrils with high trivalent cross-link density have been shown to exhibit a more "brittle-like" behavior [79]. This published information corroborates the suggestion that the elevated Pyd content contributes to the bone fragility in the Cases group. Previous studies have shown type I collagen orientation to be strongly associated with low-trauma fracture incidence [71,80]. The Raman peaks used in the present study are tissue organization/orientation independent, thus it is not possible to compare our present results with the previously published ones. On the other hand, in addition to the differences in type I collagen orientation, low-trauma sustaining patients also exhibited smaller lamellar width compared to controls [80]. Pyd content is known to inversely modulate interfibrillar-spacing [81], thus the elevated Pyd content would be in general agreement with the smaller lamella reported in [80]. Despite the fact this previous work [80] entailed consideration of non-osteoporotic women, it should be noted that osteoporotic bone (as is the case in the present study) has also been reported to have decreased mean fibril diameter and spacing [82] compared to healthy. The results would be also in general agreement with [83] reporting reduced lamellar thickness in patients sustaining fragility fractures, albeit at endocortical sites.

CML and PEN are two AGEs occurring in bone, elevated concentration of which adversely affects bone strength [35,84]. AGEs are the products of non-enzymatic glycation and oxidation of proteins and lipids [85]. In the present study, both PEN and CML were elevated in Cases compared to Controls, although only CML content differences reached statistical significance, a finding that would be consistent with the bone fragility evident in the former group.

In summary, the measurement implications from this preliminary study are that the fracturing patients have increased risk of fracture owing to the loss of normal mechanical competence of their skeletons. Indeed, the tests of their mechanical behavior (nano-indentation performed on the biopsy specimens) showed lack of heterogeneity of modulus. Interestingly, at the material level it is organic matrix rather than mineral properties that are altered in fragility-fracture sustaining patients, further highlighting the obstacles in identifying patients who are truly at risk of fragility fractures occurrence given the clinical tools currently at our disposal, in agreement with recent clinical observations [20,86].

A limitation of the present study is the fact that iliac crest biopsies were analyzed, which would be bone from a site different than the fracture one. One the other hand, iliac crest is the standard anatomical site for obtaining bone biopsies. Another limitation is the small number of patients per group analyzed, although the differences observed are consistent with previously reported ones for the analysis of larger cohorts of premenopausal idiopathic osteoporosis patients [16,52].

In conclusion, the results of the present pilot study indicate significant differences in organic matrix quality in PM women that sustain fragility fractures versus age- and BMD-matched controls, and suggest an important role as an independent determinant of fracture incidence.

Acknowledgments

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