



Cortical Fractal Analysis and Collagen Crosslinks Content in Femoral Neck After Osteoporotic Fracture in Postmenopausal Women: Comparison with Osteoarthritis

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Abstract

The femoral neck (FN) has been previously characterized by thinner cortices in osteoporotic fracture (HF) when compared to hip osteoarthritis (HOA). The purposes of this study were to complete the previous investigations on FNs from HF and HOA by analyzing the complexity of the cortical structure and to approach the intrinsic properties of cortical bone by assessing the collagen crosslink contents. FN samples were obtained during arthroplasty in 35 postmenopausal women (HF; $n = 17$; mean age 79 ± 2 years; HOA; $n = 18$; mean age 66 ± 2 years). The cortical fractal dimension (Ct.FD) and lacunarity (Ct.Lac) derived from high-resolution peripheral quantitative tomography (isotropic voxel size: $82 \mu\text{m}$) images of FN by using *Ctan* software and *FracLac* running in ImageJ were analyzed. The collagen crosslinks content [pyridinoline, deoxypyridinoline, pentosidine (PEN)] were assessed in cortical bone. Ct.FD was significantly lower ($p < 0.0001$) in HF than HOA reflecting a decreased complexity and was correlated to the age and BMD. In two sub-groups, BMD- and age-matched, respectively, Ct.FD remained significantly lower in HF than HOA ($p < 0.001$). Ct.Lac was not different between HF and HOA. PEN content was two times higher in HF than HOA ($p < 0.0001$) independently of age. In conclusion, FN with HF was characterized by a less complex cortical texture and higher PEN content than HOA. In addition to the decreased bone mass and BMD previously reported, these modifications contribute to the lower bone quality in HF than HOA in postmenopausal women.

Keywords Cortical bone · Osteoporosis · Femoral neck · Lacunarity · Fractal dimension

Introduction

Bone failure of the proximal femur occurs when the forces applied to the bone during a fall exceed its load-bearing capacity [1, 2] and this bone strength depends on the intrinsic properties, the total amount and spatial distribution of the bone tissue [3, 4]. Hip fragility is routinely predicted by the measurement of the bone mineral density (BMD) by dual-X ray absorptiometry. As the spatial distribution and the intrinsic properties of the bone tissue cannot be assessed by DXA, up to 50% of fractures occur in patients with a BMD

classified as normal according to the osteoporosis definition [5]. We have previously shown that structural changes characterize osteoporotic hip fracture. Besides a decrease of cortical and cancellous bone mass, a deterioration of the trabecular connectivity with a rod-like structure contributes to the femoral neck (FN) fracture [6, 7]. The cortical compartment plays also a major role in the FN fragility. Cortical bone has a complex microstructure, and the study of channels network is of importance in the bone quality analysis [8]. The density of channels in bone matrix is reflected by the cortical porosity (Ct.Po) but the detection of the pores is strongly influenced by the voxel size [9]. Ct.Po measured by 2D histomorphometry with a $5.6\text{-}\mu\text{m}$ pixel size resolution is not correlated with 3D measurements from high-resolution peripheral quantitative tomography (HR-pQCT) images at $82 \mu\text{m}$ voxel size [7].

Fractal geometry which has been initially used for characterizing porous materials has been applied to image analysis of bone tissue [10]. Derived from this analysis, the fractal

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dimension (FD) reflects the degree of complexity and the lacunarity (Lac) quantifies the emptiness [11]. FD indicates how much an object fills up the space, and is an indicator of the boundary irregularity and roughness. The Lac values reflect the degree of gap (or *lacuna* from Latin) distribution in a given area [12]. The Lac definition can be considered as a scale-dependent measurement of heterogeneity or texture. It reflects the similarity of the different regions of a geometric object. Objects which appear homogeneous at a small scale may become heterogeneous when examined at a larger scale, and vice versa [13]. These two parameters allow the evaluation of the 3D pattern of a structure, such as the cortical pores in cortices. *Ex vivo* studies have reported correlations between FD and mechanical properties of bone [14, 15] suggesting that FD by reflecting the microstructure may provide information on the bone quality.

In addition to the bone architecture, the intrinsic properties of the bone matrix such as changes in collagen crosslink concentrations may also contribute to FN fragility [3]. Two types of collagen cross-links coexist in bone. The enzymatic crosslinks mediated by lysyl-oxidase that generate immature divalent crosslinks which spontaneously turn into mature trivalent crosslinks such as pyridinoline (PYD), deoxypyridinoline (DPD), and pyrrole. These enzymatic crosslinking stabilize the collagen matrix of bone [16]. The other type of crosslinks derives from a non-enzymatic mechanism of glycation which forms advanced glycation products (AGEs). They include various chemical structures partly characterized, which can either only modify proteins (e.g., carboxymethyllysine and carboxyethyllysine) or form crosslinks [e.g., glucosepane and pentosidine (PEN)] [17]. PEN, an arginine–lysine-derived product, is not the most abundant AGEs but it is the most extensively studied and represents about 40% of the fluorescent AGEs that accumulate in adult bone [18]. PEN content has been reported to positively correlate with trabecular number and connectivity density [19].

The risk of hip fracture is high in the elderly resulting in a high level of morbidity and mortality [20, 21]. Thus, it is important to characterize the modifications of the different determinants of the bone quality at the site of fracture. The purposes of this study were to complete the previous investigations on FNs after non-traumatic fracture and from osteoarthritic patients by analyzing the complexity of the cortical structure and to approach the intrinsic properties of cortical bone by assessing the collagen crosslink contents.

Materials and Methods

Subjects

Postmenopausal women included in the study have suffered from either a non-traumatic intracapsular fracture of the FN

resulting from a fall from less than standing height (HF; $n = 17$; mean age 80 ± 2 years) or a hip osteoarthritis (HOA; $n = 18$; mean age 66 ± 2 years). Women with a previous history of disease known to affect bone metabolism (e.g., carcinoma or long-term treatment with glucocorticoids) were not included. HF patients were naïve for osteoporosis treatment and did not receive corticosteroid for more than 6 months at doses equivalent > 7.5 mg prednisone during the last 5 years. Written informed consent to participation in the study, which was approved by the research ethics committee, was obtained for all women.

Bone Mineral Density

In vivo measurements of BMD of the FN injured by HOA and of controlateral FN to the prosthesis in HF women were performed by dual-photon X-ray absorptiometry using a Lunar DPX unit (Lunar Corporation, Madison, WI, USA) and Lunar version 3.6 software.

Bone Histomorphometry

As previously described, the FN samples were embedded in methyl metacrylate without prior decalcification. Serial 10- μm -thick sections were cut at three different levels separated by at least 800 μm and stained with Goldner trichrome [6]. Parameters of bone structure and microarchitecture [cortical thickness (Ct.Th) and Ct.Po] were obtained with Bone software (Explora Nova, La Rochelle, France) specific for the bone histomorphometry.

HR-pQCT and Fractal Analysis

Three-dimensional bone microarchitecture of embedded FN was measured using a HR-pQCT system (XtremeCT, Scanco Medical AG®, Brüttisellen, Switzerland), with a nominal isotropic voxel size of 82 μm , using the standard in vivo scanning protocol (60 kVp, 900 μA) [7].

Image processing included a Gaussian filter (support = 1, $\sigma = 0.8$). Cortical 3D parameters were analyzed using *CTan* software V1.13.12.0 (Skyscan, Belgium). The global threshold used for bone segmentation (0.60 g/cm^3) was chosen to differentiate the Ct.Po (Fig. 1). The following cortical 3D parameters were assessed: Ct.Th, cortical thickness heterogeneity, Ct.Po and cortical fractal dimension (Ct.FD). The gray-scale images were analyzed by lacunarity (Ct.Lac) using the Box Counting method [22–24]. This method consists in randomly chosen boxes over the figure and in checking whether they are filled and analyzed on range in intensity over all pixels in a box at different box sizes. Lac was assessed during fractal analysis measuring the degree of inhomogeneity and translational and rotational invariance in the images. The method was performed with two

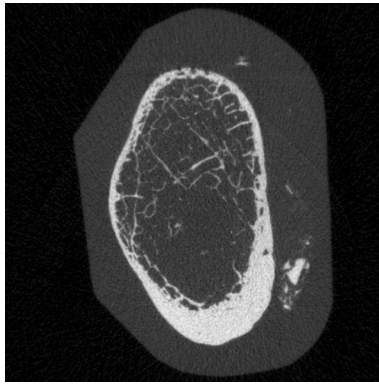


Fig. 1 Cross section of a HF FN:HR-pQCT gray-level slice of the sample

scan positions and the *differential* type of scan. The analyses consist in the difference or range in intensity over all pixels in a box at a box size. The selection of the region of interest (ROI) included the evaluation of a stack composed by all HR-pQCT images of each sample. To define the stack, the ROI was selected in each layer and finally grouped forming a volume. The analysis of all layers in the volume provides Ct.Lac value.

Collagen Crosslinks Content in Bone

To determine levels of PYD, DPD, and PEN, 20- μ m-thick sections were cut from the embedded FN with a precision diamond wire saw [25]. Each bone section was incubated at room temperature, under gently stirring in three successive methyl methacrylate baths during 3 days to dissolve polymerized methyl methacrylate, washed with ethanol and progressively rehydrated in graded alcohol/water baths. Then cortical of each section was finely separated and hydrolyzed in 6 N hydrochloric acid during 20 h at 110 °C. Collagen crosslinks were extracted from acid hydrolysate using Solid Phase Extraction Chromatography (Chromabond® Crosslinks, Macherey Nagel, Düren, Germany), separated by HPLC using an Atlantis dC18, 3 μ m, 4.6 \times 100 mm reversed phase column (Waters Corp., Milford, MA, USA) and quantified by fluorescence as previously reported. The amount of PYD, DPD, and PEN were expressed in millimole per mole of collagen determined by hydroxyproline HPLC assay (Biorad, Munchen, Germany) [26].

Statistical Analysis

Results were expressed as mean \pm standard error of the mean (SEM). Non-parametric tests were performed because the distributions were not normal. The comparisons between HOA and HF groups were performed by unpaired Mann–Whitney *U* test. Correlation tests were performed by

Table 1 Characteristics of postmenopausal women with HOA or HF

	HF (<i>n</i> = 17)	HOA (<i>n</i> = 18)
Age (years)	79 \pm 2	66 \pm 2**
Body weight (kg)	62 \pm 3	71 \pm 4
Height (cm)	160 \pm 1	165 \pm 1 ^a
Lean mass (kg)	36.9 \pm 1.7	42.4 \pm 1.3
Body mass index (kg/m ²)	24.63 \pm 1.13	26.21 \pm 1.09
BMD _{Ward} ^a (g/cm ²)	0.41 \pm 0.04	0.59 \pm 0.04 ^{b,*}
BMD _{Trochanter} ^a (g/cm ²)	0.52 \pm 0.02	0.63 \pm 0.03 ^{b,*}
BMD _{Neck} ^a (g/cm ²)	0.61 \pm 0.03	0.77 \pm 0.03 ^{b,*}
T-score _{Neck} ^a	-2.47 \pm 0.31	-0.77 \pm 0.37 ^{b,*}

p* < 0.005; *p* < 0.0001

^aBMD was measured on the opposite side of hip arthroplasty in the HF group and homolateral in the HOA group

^bBMD could be measured only in 10 HF patients. HF versus HOA (Mann–Whitney *U* test)

Spearman test. Results were considered significant when *p* < 0.05. All statistical analyses were performed using IBM SPSS Statistics 22 software (IBM, USA).

Results

The details of subjects' age, body lean mass, and BMD are given in Table 1. Women with HF were significantly older than those with HOA, and had a lower FN T-score.

Fractal Analysis

Ct.FD was highly significantly lower in HF than HOA group (*p* = 0.0001) (Table 2), and Ct.FD was significantly and negatively correlated with the age (*r*' = -0.59, *p* = 0.0001) (Table 3). As the age was significantly different between the two groups, the comparison was performed in 15 age-matched patients [HF (*n* = 8) aged 74 \pm 6 years; HOA (*n* = 7) aged 72 \pm 7 years]. In these sub-groups, Ct.FD remained highly significantly lower in HF than HOA (*p* = 0.001) (Table 4).

Ct.FD was significantly and positively correlated with BMD_{Neck} (*r*' = 0.46, *p* = 0.02) (Table 3). In order to evaluate the influence of the BMD_{Neck} on the decrease of Ct.FD in HF, 18 BMD_{Neck}-matched patients were also analyzed [HF (*n* = 10) BMD_{Neck}: 0.61 \pm 0.10 g/cm³; HOA (*n* = 8) BMD_{Neck}: 0.65 \pm 0.04 g/cm³]. In these sub-groups, Ct.FD remained highly significantly lower in HF than HOA (*p* = 0.001) (Table 4). Ct.FD was also significantly correlated with the structural parameters Ct.Th_{2D}, Ct.Th_{3D}, and Ct.Po_{2D} (Table 3).

In contrast, Ct.Lac did not significantly differ between the two groups (Tables 2 and 3) and no correlation was found

Table 2 Cortical analysis and collagen crosslinks content in the FN in HF and HOA postmenopausal women

	HF (n = 17)	HOA (n = 18)	p*
Ct.FD	2.11 (0.01) ^a	2.57 (0.01)	0.0001
Ct.Lac	1.71 (0.22)	1.54 (0.22)	0.50
Ct.Po _{2D} (%)	8.9 (1.1)	13.8 (1.1)	0.002
Ct.Th _{2D} (μm)	1450 (114)	2002 (108)	0.001
Ct.Po _{3D} (%)	19.15 (1.62)	16.96 (1.51)	0.53
Ct.Th _{3D} (μm)	1511 (69)	1608 (68)	0.41
PYD ^b (mmol/mol col)	587.15 (22.23)	581.43 (17.84)	0.80
DPD ^b (mmol/mol col)	178.31 (10.65)	187.36 (9.28)	0.40
PEN ^b (mmol/mol col)	47.73 (4.79)	24.86 (3.34)	0.0001
PYD/DPD ^b	3.40 (0.18)	3.17 (0.13)	0.54

Bold values are statistically significant

^aMean (SEM)

^bCollagen crosslinks could be measured only in 14 HF and HOA patients

*Mann–Whitney *U* test

between Ct.Lac and the other parameters of cortical structure (Table 4).

Collagen Crosslinks Content

Collagen crosslinks content could be assessed in 14 patients in both HF and HOA. Ct-PEN content in cortical FN was twice higher in HF than HOA ($p = 0.0001$) (Table 2) and remained significantly higher in both the age- and BMD_{Neck}-matched groups ($p < 0.02$) (Table 4). Ct-PEN was significantly and negatively correlated to Ct.FD ($r' = -0.61$; $p = 0.001$). PYD and DPD contents were similar in HF and HOA (Table 3).

Discussion

Besides decreased BMD, bone mass and porosity, the cortices of HF FNs were characterized by a lower Ct.FD when compared to HOA showing that the bone fragility of FN was associated with a diminution of the cortical complexity. This was in agreement with previous studies performed in trabecular bone which found a positive correlation between

FD and mechanical properties [10, 15, 26]. An increase in FD is correlated with an increase in the failure load i.e., in the bone strength [15]. Ct.FD being also significantly linked to the age, the diminution of Ct.FD in HF group may be only related to the age of HF patients who were 13 years older than HOA patients. In a sub-group of aged-matched patients, Ct.FD remained significantly lower in HF than HOA group showing that the decreased cortical complexity is one determinant of the bone fragility independent on the aging. Similar observations were made regarding the influence of BMD on Ct.FD. The non-traumatic fractures in HF group resulted from osteoporosis as shown by a mean T-score of -2.5 . However, in HOA group, the BMD of some patients was similar to HF patients. Despite a same BMD, Ct.FD was significantly lower in HF than HOA groups. Thus, the complexity of the FN cortices evaluated by Ct.FD contributes to the bone strength as it has been previously reported for the cancellous compartment. The cancellous bone strength has been shown more dependent on the complexity of the trabecular structure than on the bone volume [27] and FD in cancellous bone is lower in osteoporotic patients with vertebral fracture than controls [28].

In contrast, the Lac, which reflects the distribution of the pores, is not different in HF and HOA FNs. It is in agreement with our previous results reporting a similar Ct.Po_{3D} in HF and HOA when assessed by HR-pQCT [7]. In contrast, Ct.Po_{2D} evaluated by histomorphometry is significantly lower in HF than HOA [6]. This apparent discrepancy was due to the difference of pixel size resolution between the two methods. Zebaze and Seeman [29] reported that only pores over 130 μm in diameter could be detected by using HR-pQCT with 82 μm voxel size. Due to the used method, the resolution strongly limits the identification of pores, as their mean diameter is about 60 μm in aged women [30], with 60% of them are < 100 μm [29]. Microscopic observation allows the identification of structures lower than 5 μm and the detection of smaller pores than HR-pQCT.

Any modification of one of the determinants of the bone quality may induce the occurrence of fragility fracture [3, 4]. Variations of the intrinsic properties of bone tissue such as the collagen crosslinks must be also investigated. We found a higher PEN content in HF FN than HOA. It has been shown that with aging, an accumulation of AGEs in bone tissue occurs and modifies the physiological properties of collagen

Table 3 Correlation coefficients of fractal parameters with DXA, parameters of structure and PEN

	Age (n = 35)	BMD _{Neck} (n = 28)	Ct.Po _{2D} (n = 35)	Ct.Th _{2D} (n = 35)	Ct-PEN (n = 28)
Ct.FD	$r' = -0.59^a$ $p = 0.0001$	$r' = 0.46$ $p = 0.013$	$r' = -0.37$ $p = 0.029$	$r' = -0.61$ $p = 0.0001$	$r' = -0.61$ $p = 0.001$
Ct.Lac	$r' = -0.23$ $p = 0.17$	$r' = -0.26$ $p = 0.17$	$r' = -0.07$ $p = 0.70$	$r' = -0.11$ $p = 0.50$	$r' = -0.03$ $p = 0.89$

^aSpearman coefficient of correlation

Table 4 Comparisons between HF and HOA FNs in BMD_{Neck}- and age-matched groups

BMD _{Neck} -matched groups	HF (n = 10)	HOA (n = 8)	p*
Age (years)	81 (2) ^a	67 (3)	0.003
BMD _{Neck} (g/cm ²)	0.61 (0.03)	0.65 (0.02)	0.35
Ct.Th _{2D} (μm)	1525 (189)	1821 (132)	0.15
Ct.Po _{2D} (%)	9.31 (1.63)	11.49 (1.56)	0.15
Ct.Th _{3D} (μm)	1527 (97)	1581 (93)	0.83
Ct.Po _{3D} (%)	17.83 (1.98)	15.44 (2.59)	0.46
Ct-FD	2.11 (0.02)	2.59 (0.02)	0.0001
Ct-Lac	1.69 (0.22)	1.74 (0.28)	0.86
Ct-PEN (mmol/mol col)	48.68 (3.23)	21.20 (6.67)	0.01
Age-matched groups	HF (n = 8)	HOA (n = 7)	p*
Age (years)	74 (2)	72 (3)	0.68
BMD _{Neck} (g/cm ²)	0.60 (0.07)	0.77 (0.05)	0.07
Ct.Th _{2D} (μm)	11.49 (1.56)	9.31 (1.63)	0.15
Ct.Po _{2D} (%)	11.11 (1.75)	17.22 (1.41)	0.01
Ct.Th _{3D} (μm)	1544 (79)	1590 (72)	0.78
Ct.Po _{3D} (%)	19.32 (1.09)	19.97 (2.24)	0.61
Ct-FD	2.10 (0.02)	2.54 (0.02)	0.001
Ct-Lac	1.62 (0.43)	1.67 (0.49)	0.99
Ct-PEN (mmol/mol col)	44.13 (4.49)	28.71 (4.53)	0.02

Bold values are statistically significant

^aMean (SEM)

*Mann–Whitney *U* test

[31]. However in our present study, the difference in the PEN content between the two groups was observed in the age-matched sub-groups. Previous studies suggested that AGEs formation makes the collagen fibers brittle and consequently may reduce the bone strength and favor the accumulation of microdamages [32, 33]. Furthermore, PEN content in human tibia cortex predicts the initiation and progression of fracture independently of age [34].

The present results must also be interpreted within the context of a significant difference in age between HF and HOA women, which is representative of the mean age of women suffering from these two pathological conditions. The differences in Ct.FD and PEN between the two groups cannot only be explained by the difference in age of the women, the comparison in the sub-groups confirming the results observed in the whole group.

Some limitations may be addressed regarding the methods used and the patients analyzed. First, the determination of Ct.FD was made by HR-pQCT on FN which cannot be performed in vivo. Recent studies have shown that in vivo measurements acquired with high-resolution multi-detector CT strongly correlate with those assessed using either μCT or HR-pQCT [35, 36]. Second, FN samples being embedded in methyl methacrylate, we have previously checked the effects of embedding process on the crosslinks

content. No significant difference of PYD, DPD, and PEN concentrations was observed in embedded compared to non-embedded bone sections (data not shown). However, the labile divalent immature enzymatic crosslinks were fully destroyed by the embedding process and could not be quantified. Therefore, the relationship between mature and immature crosslinks which may contribute to the bone strength [37] could not be assessed. PEN, the only measured non-enzymatic crosslinks, is known to be an interesting surrogate marker of the accumulation of AGEs in bone tissue [17, 18]. Third, the present study investigated cortical FN of women with hip osteoporotic fractures against women with HOA. The patients in this latter group cannot be considered as true controls, even though they are non-osteoporotic women (T-score_{Neck}: -0.77 ± 0.37). This limitation can be considered as relatively minor, the main goal of the study being to better characterize the texture and the intrinsic properties of cortical bone at a specific site of fracture. The recruitment of true controls, which would require the collection of FN bone samples from age- and sex-matched cadavers, is difficult and somewhat questionable. Cadavers cannot be considered as suitable controls, most of them being very old probably with a much reduced physical activity during the last years of their life and their health conditions are unknown.

In conclusion, despite these limitations, our data show that, when compared with subjects with HOA, bone fractal analysis reveals a distinct structural pattern which, associated with an accumulation in PEN, contribute to the bone fragility in postmenopausal osteoporotic women with a FN fracture.

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Author Contribution GDR and PC designed the study and were evolved in all phases of the study and in writing the manuscript. JPR, NPM, and EG contributed to the experimental work and drafting the results. GDR, JPR, and PC were responsible for statistical analysis of the data. RC was responsible for the discussion of the results and supervision. All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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