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Changes in bone microarchitecture in rheumatoid arthritis. Study using microCT

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Date of receipt: 15/02/2010
Date of acceptance: 16/06/2010

Summary
Introduction: The objective of this study is to analyse the bone microarchitecture in rheumatoid arthritis (RA) in a series of biopsies of the iliac crest carried out previously in patients not having had earlier treatment with glucocorticoids, using microCT analysis.
Material and method: 14 bone specimens were obtained, taken from the iliac crest of patients with RA with no previous treatment with glucocorticoids. None of these patients was diagnosed with a disease or was taking medicines which could compromise bone mineral metabolism. A complete clinical history was taken, and a blood analysis carried out, including the rheumatoid factor. The specimens were embedded in methyl-methacrylate and studied with a microCT eXplorer Locus SP scanner. The acquisition parameters were: 80 kVp/80 μA, thickness of aluminium filter:10^{-3} inches, FOV ≈ 2x2 cm, mode of acquisition of 360°, 720 views, 4 frame averages/view, exposure time 1.700 ms, voxel resolution: 28 μm. A region of interest (ROI) was selected by means of interpolation, avoiding cortical bone. An automatic segmentation process (thresholding) was used to differentiate and segment the hematopoietic bone tissue. The microarchitectural parameters were generated automatically by computer using parallel-plate algorithms. The results were compared with 14 specimens from healthy controls of similar age and sex using Student's test for unpaired samples. The statistical significance was $p< 0.05$.
Results: The fraction of bone volume (BV/TV) was significantly lower in those patients with RA than in the healthy controls ($p< 0.05$). The trabecular thickness (Tb.Th) was higher in the controls. The trabecular separation (Tb.Sp) was higher in those specimens with RA ($p< 0.05$). The trabecular connectivity (Tb.N) was significantly greater in the control specimens ($p< 0.05$).
Conclusions: The patients with RA have worse trabecular bone quality and low trabecular connectivity. The microCT scanner is a quick and powerful tool for the study of trabecular microstructure.

Key words: *Rheumatoid arthritis, Microarquitecture, Bone, Computed Tomography.*

Introduction

Osteoporosis is a global health problem[1]. It has been defined by the National Institutes of Health as "a disease characterised by low bone mass and a deterioration in the microarchitecture of bone tissue which drives an increase in bone fragility and a consequent increase in the risk of fracture"[2]. It is for this reason that alterations in trabecular bone are not only characterised by reductions in bone mineral density (BMD), but also by changes in bone quality, a term which encompasses microarchitecture, bone turnover, microfractures and bone mineralisation[3].

Rheumatoid arthritis (RA) is a chronic inflammatory disease with autoimmune origins and unknown etiology which mainly attacks the synovial joints, producing arthritis. In patients with RA, reductions in BMD have been described in two forms: juxta-articular osteoporosis (one of the earliest findings) and generalised osteoporosis, in locations distant from the inflamed joints. To date, different series of patients with RA have been described with a great prevalence for generalised osteoporosis[4-13,34], as well as an increase in the risk of fracture[14-16]. The factors most determinant of bone loss in these patients appear to be a reduction in physical activity in the most advanced stages of the disease[10,11,17], as well as chronic treatment with glucocorticoids[7-9,18,19]. In addition, low levels of vitamin D have been associated with prolonged periods of confinement to bed, with those with very limited functionality, and with diets poor in calcium[20,21,39,40]. On the other hand, in recent years there is more and more discussion regarding the role played by pro-inflammatory cytokines such as TNF-α and IL-1, which have been shown to increase osteoclast resorption by the differentiation of synovial microphages into osteoclasts[22-26].

To date, few histomorphometric studies have been carried out in patients with RA. Mellish et al. studied 48 bone specimens from patients with RA who had not been treated with corticoids, finding a lower fraction of bone volume and a lower trabecular thickness than in the controls, findings which suggest that RA not treated with steroids is associated with premature bone loss. These results were only significant in women[35]. Pérez-Edo et al. described an association between hypovitaminosis D and a reduction in bone turnover in transiliac bone biopsies of patients with RA, confirming findings published by Compston et al. in 1994[21,44]. Hanyu et al. found a reduction in trabecular thickness and in bone connectivity in menopausal patients with RA compared with controls of similar age with osteopenia[45]. Laan et al., on their part, studied different cohorts of patients with RA treated with steroids, finding a lowering of cortical and trabecular BMD in the lumbar spine, which was partially reversible by the interruption of the corticoid treatment[29-31]. Summarising, it appears that the decrease in bone mass in patients with RA is of multifactorial etiology, notable among which being the effect of the pro-inflammatory cytokines and prolonged treatment with glucocorticoids. Despite the fact that conventional histomorphometry allows us to identify this type of osteoporosis, it is an invasive examination, which makes the search for non-invasive alternatives a fundamental objective. Except for the conventional histomorphometric studies, to date no studies have been published which have specifically looked at the trabecular microarchitecture in osteoporosis through multiplanar three-dimensional techniques such as micro-CT or p-QCT (Peripheral Quantitative Computerised Tomography), techniques which allow the measurement of the trabecular (and cortical) microarchitectural parameters in the radius and distal tibia in a non-invasive way[28].

The principal objective of this study is to evaluate the discriminative capacity of microCT to differentiate between patients with RA but without corticoid treatment and healthy controls using previously carried out biopsies of the iliac crest. These bone specimens come from the documentary records of biopsies of the pathological anatomy service of the Hospital del Mar. Our hypothesis holds that the bone samples of those patients with RA will show a deterioration in their bone quality.

Material and method

A total of 66 patients who met the 1987criteria of the American Rheumatism Association for the diagnosis of RA[36] were randomly chosen from the totality of patients of the rheumatology service of the Hospital del Mar and the Hospital de la Esperanza in Barcelona. None of these patients had other diseases or were taking any medicine with could affect bone metabolism, with the exception of 22 patients who were receiving oral corticoid treatment at low doses (< 8 mg/d of prednisone) over a period of 47 ± 61.8 months (range 6-240 months), with an accumulated dose of 6.34 ± 8.76. The remaining patients (44) had never started corticoid treatment. All the patients were in treatment with non-steroidal anti-inflammatories (AINEs) and 67% were receiving treatment with anti-rheumatic drugs of the DMARD (Disease-Modifying Anti-Rheumatic Drugs) type. The same diagnostic protocol was carried out in all patients, which included a complete clinical history, with a particular emphasis on the existence of diseases which might affect bone metabolism and the use of drugs toxic to bone tissue.

A complete biochemical profiling was carried out, including parameters for inflammatory activity. The degree of functionality was measured by means of the Steinbrocker functionality index[37]. The BMD was measured in the lumbar spine using densitometry (DXA)[38] in 41 patients (34 women and 7 men) using a Hologic QDR-1000 (Hologic Inc. Waltham, MA, USA) densitometer. The precision of the apparatus is 0.45% with an *in vivo* coefficient of variation of 1.2% in the lumbar spine.

The most significant clinical and epidemiological data of all the patients with RA initially chosen for the study are shown retrospectively in Table 1.

Performance of bone biopsy: fourteen bone biopsies were obtained from patients (4 men, 10 women), diagnosed with RA without receiving glucocorticoidal treatment, from the documentary records of biopsies of the pathological anatomy service. These bone specimens are the same as those used by Pérez-Edo et al.[21] in their study. There were no significant differences between these patients and the rest of the patients who had followed steroid treatment in terms of age (59.1 ± 10.7 vs 59.9 ± 12.6 years; $p< 0.05$) and the Steinbrocker index (2.2 ± 0.6 vs 2.4 ± 0.6; $p< 0.05$). Each transiliac bone biopsy was obtained using local anaesthetic with a Bordier-Meunier trepan of 8 mm interior diameter (Lepine, Lyon-Cedek, France)[32]. Each specimen was fixed in 70% ethanol, dehydrated in decreasing concentrations of ethyl alcohol and embedded in a cylinder of methyl-methacrylate of 2 cm diameter. Sections 5 μm thick were obtained by mycrotomy (Supercut2050, Reichert Jung, Germany), subsequently stained with Von Kossa's stain and Goldner´s trichrome.

Finally the following histomorphometric statistical parameters were calculated: trabecular bone volume (BV/TV_H; %) and average trabecular thickness ($Tb.Th_H$; μm) by direct microscopic measurement. Derived parameters such as average trabecular density ($Tb.N_H$; μm^{-1}) and average trabecular separation (Tb.SpH; μm) were calculated according to the following formulae[22]:

$$Tb.N = (BV/TV)/Tb.Th$$

$$Tb.Sp = (1/Tb.N) - Tb.Th$$

We described retrospectively the histomorphometric values obtained from the 14 specimens with RA: BV/TV_H (%): 13.52 ± 5.39; $Tb.Th_H$ (μm): 152.44 ± 37.87; $Tb.Sp_H$ (μm): 1157.3 ± 639.84 and $Tb.N_H$ (μm^{-1}): 0.8650 ± 0.2617.

Acquisition of images using microCT: The bone specimens embedded in methyl-methacrylate were introduced into a sample cylinder, and secured with a strip of polyethylene foam to ensure their immobilisation. The capture of the images was carried out with the microCT for specimens eXplore Locus SP (GE Healthcare). The data was collected using the following parameters: voltage of tube: 80 kVp; current of tube: 80 μA, thickness of aluminium filter: 0.010 inches, FOV ≈ 2x2 cm^2 depending on the size of the specimen, mode of acquisition in 360°, 720 views (projections), increment of 0.5° between each projection, 4 images/projection, exposure time: 1,700 ms. The scanning time for each specimen was approximately 2 hours, plus time for reconstruction of 1 hour.

The volumetric data were reconstructed to a resolution of 28-μm isotropic voxels (2.2 x 10^{-5} mm^3 per voxel) using Feldkamp's conical algorithm. 28-μm was chosen to improve the signal-noise quotient of the images obtained, to reduce the scanning time and to lessen the volume of data obtained. The analysis of the images and the generation of the microarchitectural parameters were carried out using MicroView© (GE Healthcare) software.

Due to the fact that the volume of trabecular bone tends to vary, especially sharply decreasing towards the endosteal surface, the region of interest (ROI) to be quantified was selected in trabecular bone using two different methods: one restricting the analysis to only the central trabeculae of the biopsied sample, and the other including all the trabeculae from the endosteal surface. The first method used a cylindrical ROI aligned parallel to the external cortical surfaces with a diameter exactly 50% of the distance between both endosteal surfaces. In the second method a curved outline (spline fitting drawing) which encompassed the combination of trabeculae in each of the cuts was used, the ROI being created subsequently through interpolation. The cortical bone was excluded from the analysis in both methods. In order to avoid artefacts the study of sections or slices near to the edges of the cut were omitted.

For each ROI the bone tissue was segmented from the bone medulla by means of a software application which differentiates the intensity of each of the voxels (bimodal histogram thresholding). The microarchitectural parameters BV/TV_{CIL}, $Tb.Th_{CIL}$, $Tb.Sp_{CIL}$ and $Tb.N_{CIL}$ (for cylindrical ROI) and BV/TV_{SPL}, $Tb.Th_{SPL}$, $Tb.Sp_{SPL}$ and $Tb.N_{SPL}$ (for curved ROI) were calculated automatically using the same parallel-plate algorithms mentioned earlier for conventional histomorphometry, recalculating that Tb.Th was determined by means of an image-processing algorithm included in MicroView©. The stages in the acquisition and processing of the images are summarised in Figure 1.

The Euler-Poincaré number and the Euler volume ($EulerV_{CIL}$ and $EulerV_{SPL}$) were also calculated. All the histomorphometric and microCT results of the patients with RA were compared with a control group of similar sex and age made up of bone biopsies from 14 healthy donors from the documentary records of biopsies from the pathological anatomy service.

Statistical analysis: The data were compiled on a spreadsheet (Microsoft Excel 2002) and were analysed statistically using JMP software (version 5.1.2, SAS Institute Inc. Cary, NC, USA). A basic descriptive statistical study was carried out, applying the Shapiro-Wilk normality test for continuous variables (sample size ≤ 2,000). The statistical significance was set at $p< 0.05$, and the results were expressed as an average ± SD. The comparisons of the microarchitectural data obtained in patients with RA and in healthy donors were carried out using the Student's t test unpaired for multiple comparisons.

Results

In the end, a total of 14 bone samples were included from 10 women and 4 men. No significant differences were found in age between the patients and healthy donors ($p< 0.05$). Even though the volume of the ROIs generated was between 5 and 5.4 times greater in the curved outline than in the cylindrical, a close concordance between both

Table 1. Clinical and epidemiological data for all patients with RA (n= 66)

	Men (n=16) Average ± SD (range)	Women (n=50) Average ± SD (range)
Age, years	57.6 ± 12.2 (34-74)	60.5 ± 11.9 (33-85)
Years since the menopause; n=43	---	16 ± 9.6 (0-45)
Body mass index	24.9 ± 3.1 (18.1-32.5)	25.5 ± 4 (16.8-38)
Duration of the disease, months	68.1 ± 78.05 (6-240)	84 ± 87.6 (6-360)
Prednisolone, accumulated dose (g)	12.0 ± 14.2 (1.1-34.5)	5.3 ± 7.3 (0.7-28)
Steinbrocker's index	2.1 ± 0.5 (1-3)	2.4 ± 0.5 (1-3)
Rheumatoid factor (IU/mL)	521 ± 640 (69-2015)	353 ± 514.2 (65-2150)
VSG (mm/1ºhour)	31.3 ± 16.6 (4-60)	41.7 ± 23.2 (7-85)
PCR (mg/dL)	1.2 ± 0.9 (0.5-3.6)	2.9 ± 2.8 (0.5-10.2)
25-OH-vitamin D, ng/mL	12.57 ± 14.9 (2.2-48.9)	7.45 ± 5.62 (1.5-33.6)
Lumbar DXA (g/cm^2)	0.764 ± 0.118 (0.491-0.935)	0.592 ± 0.129 (0.091-0.792)

methods (ROICIL and ROISPL) was found for all microarchitectural parameters (r^2= 0.83-0.91) especially for Tb.Sp and Tb.N (r^2= 0.91). All the results obtained through microCT in patients and controls for each model of ROI are described below in Table 2.

The trabecular bone mass measured by the two methods (BV/TV_{CIL} and BV/TV_{SPL}) in patients with RA was significantly lower than in the control group (p< 0.05). $Tb.Th_{CIL}$ in the specimens with RA was lower than in the control group, but this difference was not significant (p= 0.83). At any rate, in calculating $Tb.Th_{SPL}$ the result was on the margin of statistical significance (p= 0.06), probably due to the inclusion of thicker peripheral trabeculae at the time of the selection of the ROI. It is probable that this justifies the slightly (although not significantly) higher values for BV/TV and Tb.Th when the same specimen was analysed using both models of ROI selection.

As was expected, those specimens with RA obtained higher values for $Tb.Sp_{CIL}$ and $Tb.Sp_{SPL}$ than the healthy controls (p= 0.028 and p= 0.013, respectively). TbN, or trabecular number, is a parameter which represents the average number of trabecuale per μm. A expected, the controls had higher values for this parameter, but this difference was only statistically significant when the curved ROI model was used (p= 0.027). The volumetric reconstructions of three cylindrical bone cores from patients with RA and from one control exemplify visually the predominance of the trabecular structure in each group (Figure 2).

As has been mentioned earlier, the Euler volume measures the connectivity by unit of volume. As expected, the healthy controls had Euler volumes higher than the patients with RA in both models (EV_{CIL} p< 0.01; EV_{SPL} p= 0.031). In the specimens with RA, a moderate-to-high relationship was found between EV_{SPL} and $Tb.N_{SPL}$ (r^2= 0.69; p< 0.01).

Discussion

To date, earlier studies have clearly demonstrated the association between RA and a reduced bone mineral density, but none had examined directly the microarchitectural changes in humans using a technique such as microCT. In this study we have used this three-dimensional technique to determine whether the trabecular microstructure in transiliac biopsies of patients with RA differ from those from healthy donors. The results show a lower fraction of trabecular bone volume (BV/TV) and a lower average trabecular thickness (Tb.Th) in the specimens with RA in comparison with the controls, as well as

Table 2. Structural parameters of the patients with RA and their controls measured using microCT

	Parameter		RA (n=14)	Controls (n=14)	p*
ROI cylindrical (ROI_{CIL})	Volume of ROI (mm^3)	Total	23.55 ± 11,5	52.47 ± 53.47	
		Only bone	4.33 ± 2.51	13.29 ± 12.68	
	BV/TV_{CIL} (%)		18.92 ± 6.29	25.15 ± 7.46	0.024
	$Tb.Th_{CIL}$ (μm)		123.33 ± 16.91	136.46 ± 21.41	0.083
	$Th.Sp_{CIL}$ (μm)		593.84 ± 249.91	426.73 ± 99.72	0.028
	$Tb.N_{CIL}$ (μm^{-1})		1.52 ± 0.43	1.82 ± 0.31	0.051
	EV_{CIL}		3.15 ± 2.24	5.94 ± 2.42	0.004
ROI curved (ROI_{SPL})	Volume de ROI (mm^3)	Total	154.52 ± 108	154.52 ± 108	
		Only bone	20.90 ± 8.13	40.26 ± 26.4	
	BV/TV_{SPL} (%)		20.44 ± 6.22	27.45 ± 7.27	0.011
	$Tb.Th_{SPL}$ (μm)		130.04 ± 13.68	140.29 ± 13.97	0.060
	$Th.Sp_{SPL}$ (μm)		554.95 ± 203.51	393.03 ± 101.86	0.013
	$Tb.N_{SPL}$ (μm^{-1})		1.56 ± 0.40	1.95 ± 0.47	0.027
	EV_{SPL}		83.33 ± 2.66	6.92 ± 5.28	0.031

*Significant if $p < 0.05$

a higher average trabecular separation (Tb.Sp) for both models of ROI selection. With respect to the parameters for connectivity, the control specimens showed higher values of trabecular density and Euler volumes. These findings are consistent with the existence of an advanced trabecular osteoporosis in these patients free of treatment with steroids, and reflect an altered bone quality and poor trabecular connectivity related to the continuous inflammatory stimulation which occurs in RA.

It was decided to select two different ROIs (cylindrical and curved) for each specimen since in earlier publications there was no preference for either of the two types. The two ROI models were useful, but the curved outline achieved higher levels of statistical significance due to the inclusion of the entire trabecular volume (that is to say, the analysis was not restricted only to the core of the biopsy). This fact suggests that the central trabecular region of the cylindrical ROI does not adequately reflect the microstructural changes in osteoporosis, and that the peripheral trabecular regions should be considered in this type of study since they are also important when studying the microarchitecture.

However, our greatest limitation in this study was the small number of patients and controls, due to the inherent difficulty in obtaining bone biopsies. For this reason it is probable that the differences between the values of some microarchitectural parameters between the samples from patients and controls only show a statistical trend and not a clear statistical significance. This is one limitation already known in this type of study, due to the fact that this type of biopsy is difficult to obtain since patients do not tend to willingly accept invasive procedures such as a biopsy. Another significant limitation was the generation of the curved ROI based on a method of interpolation starting from a freehand selection in each of the cuts carried out. However, we believe that this is a relative problem, considering that it concerns a methodology necessary at the time to avoid the inclusion of cortical bone.

In conclusion, we believe that microCT is a relatively new imaging technique which permits a complete quantification of the trabecular microstructure, being more rapid than conventional histology and permitting a non-destructive examination of the bone specimen before the pathological analysis. Nevertheless, perhaps the most important of its limitations is that even though it allows the non-destructive examination of a bone specimen, it remains an invasive technique for the patient which therefore, in real life, does not allow diagnosis nor

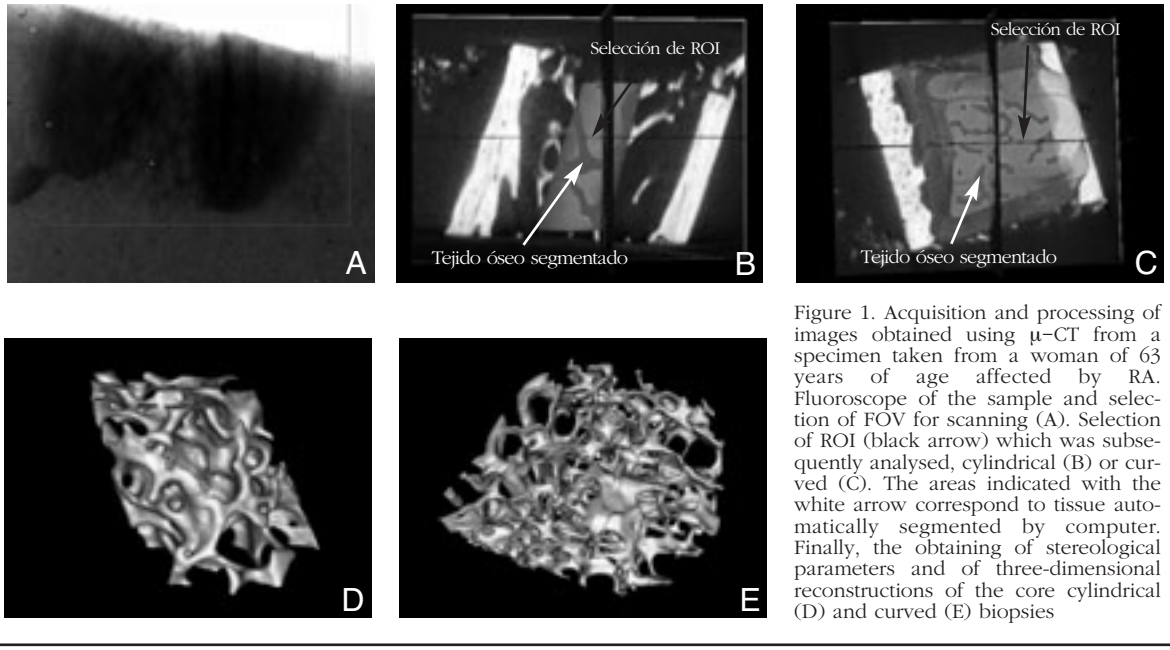

Figure 1. Acquisition and processing of images obtained using μ–CT from a specimen taken from a woman of 63 years of age affected by RA. Fluoroscope of the sample and selection of FOV for scanning (A). Selection of ROI (black arrow) which was subsequently analysed, cylindrical (B) or curved (C). The areas indicated with the white arrow correspond to tissue automatically segmented by computer. Finally, the obtaining of stereological parameters and of three-dimensional reconstructions of the core cylindrical (D) and curved (E) biopsies

post treatment follow-ups to be carried out in a routine way. Therefore we believe that an important role is played by a non-invasive technique such as p-QCT, which has been shown to distinguish, *in vivo*, between osteoporotic patients and healthy controls, to predict the risk of fracture in patients with osteoporosis, and which has demonstrated excellent correlation with results obtained by microCT[47-49]. The absence of specific studies around bone microarchitecture using p-QCT in adult patients with RA should also be mentioned.

Therefore, we have focused on the deterioration of bone microstructure in patients with RA, since: 1) they are patients who are accustomed to being treated with glucocorticoids, a fact which aggravates bone deterioration even more; and 2) the increase in the risk of fracture of the hip and/or vertebrae should be taken into account since when they occur they are a further aggravation for the patient who is already has limited functionality due to their underlying disease. Also, we believe that more studies will be required in the future due to the incidence in the population of bone fractures and their economic implications, and that these studies should be carried out using three-dimensional multiplanar techniques such as microCT or p-QCT, since both have been shown to characterise bone microarchitecture sufficiently well.

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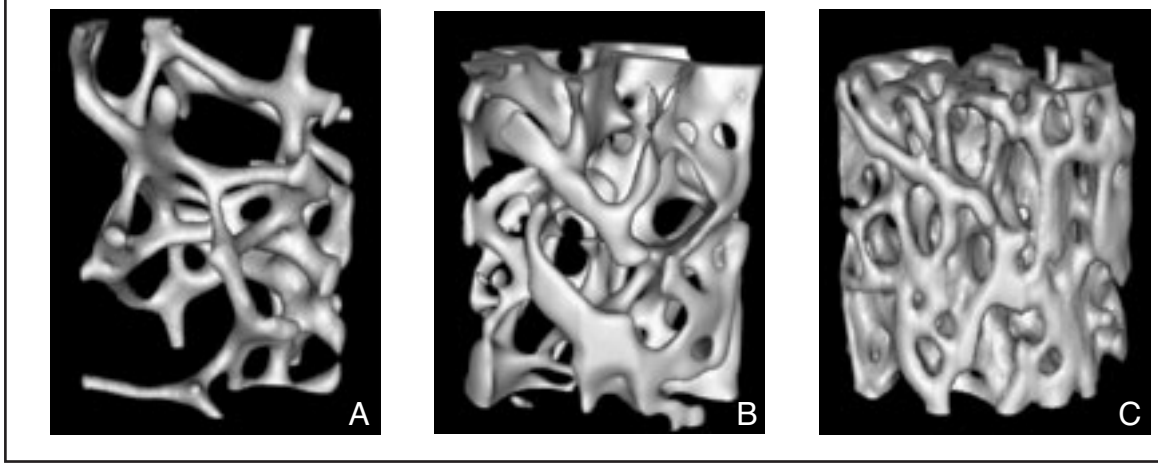
Figure 2. Trabecular microstrucutre of transiliac biopsies obtained by microCT with an isotropic resolution of 28 μm. A: cylindrical cores from patients with RA C: cylindrical core from a healthy control. The ROI volume selected was the same in the three cases. (A) Age: 68; BV/TV_{CIL}: 8.8%; $Tb.Th_{CIL}$: 118.43 μm; (B) Age: 54; BV/TV_{CIL}: 17.98%; $Tb.Th_{CIL}$: 124.59 μm; (C) Age: 38; BV/TV_{CIL}: 26.3%; $Tb.Th_{CIL}$: 137.56 μm



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