Original Article

No evidence of mineralization abnormalities in iliac bone of premenopausal women with type 2 diabetes mellitus

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Abstract

Objectives: Patients with type-2 diabetes mellitus (T2DM) have increased risk for bone fractures which points towards impaired bone quality. **Methods**: We measured bone mineralization density distribution (BMDD) and osteocyte lacunae section (OLS) characteristics based on quantitative backscattered electron images of transiliac biopsy samples from n=26 premenopausal women with T2DM. Outcomes were compared to those from reference cohorts as well as between T2DM subgroups defined by clinical characteristics. **Results**: Comparison to references did not reveal any differences in BMDD (all p>0.05) but a lowered OLS-density in cancellous bone in T2DM (-14.9%, p<0.001). Neither BMDD nor OLS-characteristics differed in T2DM subgroups defined by HbA1c (<7% versus >7%). The average degree of bone mineralization (CaMean) was higher (0.44 wt%Ca in T2DM, 0.30 wt%Ca in reference) and consistently the calcium concentration between the tetracycline double labels (CaYoung) was higher (0.76 wt%Ca, all p<0.001) in cancellous versus cortical bone. **Conclusions**: Our findings suggest that bone matrix mineralization was neither affected by the presence nor by the glycemic control of T2DM in our study cohort. The intra-individual differences between cancellous and cortical bone mineralization gave evidence for differences in the time course of the early mineralization process in these compartments in general.

Keywords: Bone Matrix Mineralization, Mineralization Processes, Osteocyte Lacunae, Transiliac Bone Biopsy, Type-2 Diabetes Mellitus

Introduction

Incidence for fractures is high in diabetes mellitus, albeit the factors leading to bone fragility appear different in type 1 and type 2 diabetes[1]. While type I diabetes mellitus is associated with a rather osteoporotic bone phenotype with low bone mineral density (BMD by DXA) and deficits in bone microarchitecture[1,2], the latter type 2 diabetes mellitus (T2DM) is generally reported to with normal or even increased BMD[3]. Thus, it seems that not bone quantity, but bone quality is altered in T2DM. It is well established that advanced glycation endproducts (AGEs) accumulation is a negative factor on mechanical properties of bone matrix[4-9]. The mineral content of bone is another factor which might essentially influence the stiffness and toughness of bone material[10] and which might be altered in T2DM[11]. However, the degree and distribution of bone matrix mineralization and their role for bone fragility in T2DM is less clear. Conflicting data were reported, ranging from normal to increased bone mineral content in T2DM[9,12-17]. Recently, mineralization data were reported obtained in a transiliac bone biopsy study from postmenopausal patients with T2DM[17]. The latter, however is an exception as most available information on this mechanically important characteristic stem from studies of femoral head samples obtained at total hip replacement surgery (thus, from patients with destructive arthrosis as co-

morbidity)[12-16].

The present work reports on the bone mineralization density distribution (BMDD)[18] and osteocyte lacunae section (OLS) characteristics[19] in T2DM patients. These were assessed based on quantitative backscattered electron imaging in tetracycline labeled transiliac bone biopsy samples which represent the standard skeletal site for the characterization of bone pathologies. Outcomes were correlated with re-visited, previously reported clinical and histomorphometric characteristics as well as serum data[20]. In a previous work, differences in the AGEs accumulation were observed between cortical and cancellous bone[9]. Thus, we compared BMDD and OLS outcomes between the two compartments explicitly. Furthermore, for information on potential differences in the mineralization kinetics of the bone matrix, we analyzed the mineral content between the tetracycline double labels.

Materials & Methods

Patients

The patients studied in this cross-sectional, observational, analytical study were n=26 premenopausal women diagnosed with type-2 diabetes mellitus (T2DM) at the Endocrinology and Metabology Service of the Federal University of Paraná (SEMPR) according to the criteria of the American Diabetes Association (ADA)[21]. Exclusion criteria of this study were secondary causes of osteoporosis, diseases and medications known to interfere with bone mineral metabolism, as well as chronic kidney disease.

A transiliac bone biopsy sample was obtained from each patient of this study. Prior to bone biopsy, patients received tetracycline hydrochloride 500 mg as follows: 3 days on, followed by a 10 days off interval and further 3 days on tetracycline. The transiliac bone biopsy was performed 3 to 5 days after the last day of label. Given this labelling schedule, the (mineralized) tissue age of bone between the double labels is 7 to 18 days.

Ethics approval

This study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and was approved by the Ethics and Research Committee of the Hospital de Clinicas, Federal University of Paraná (Opinion No. 1, 646, 120, of 7/24/2016). Informed consent to participate and to publish was obtained from the patients.

Histomorphometry

Histomorphometric structure as well as dynamic indices of bone formation of this patient cohort were reported previously[20]. In the present work, we revisited these previously obtained data for correlation analysis with BMDD, OLS as well as mineralization kinetics outcomes.

Quantitative Backscattered Electron Imaging (qBEI)

The polymethylmethacrylate embedded block samples from the previous histomorphometric study[20] were polished, and carbon coated surfaces for the qBEI analysis in the scanning electron microscope. In calibrated qBEI measurements of bone, the backscattered electron intensity (i.e., the grey level of the pixel) is proportional to the local percentage of calcium (Ca) in the bone material (the brighter the pixel, the higher the Ca content) (Figure 1). Grey level calibration and more details of the method were described elsewhere[22]. A series of qBEI images (each 1.8 mm x 1.4 mm) with nominal magnification 65x (pixel resolution 1.76 µm) covering the entire sectioned area of the bone biopsy sample was acquired and stitched for a single image (Figure 1A). For this purpose, we used a scanning electron microscope with a field emission electron source (Zeiss Supra 40, Zeiss, Oberkochen Germany) equipped with a four-quadrant semiconductor backscattered electron detector and operated at the following instrumental parameters: 20 kV accelerating voltage for the beam electrons, about 280 pA probe current, 10 mm working distance and a scan speed of 100 seconds per frame.

The bone mineralization density distribution (BMDD) was obtained from the stitched qBEI image and represents the frequency distribution of pixels with a certain Ca content (weight% Ca) of the sectioned area. Cancellous and cortical compartments were considered separately for BMDD and BMDD-parameters (Figure 1B)[23]: The weighted mean Ca-concentration of the bone area (CaMean), the peak position of the histogram (CaPeak, indicating the most frequently measured Ca concentration), the full width at half maximum of the distribution (CaWidth, describing the heterogeneity in matrix mineralization), the percentage of bone areas having a Ca-concentration lower than 18.20 wt% Ca (CaLow), and the percentage of bone areas having a Ca-concentration higher than 26.86 wt% Ca (CaHigh, corresponding to fully mineralized bone areas, mainly interstitial bone). These cut-off levels for CaLow and CaHigh correspond to the 5th and the 95th percentiles of a mean BMDD obtained from transiliac bone samples of adult bone from healthy individuals[22]. The patients' BMDD outcomes were compared to adult reference BMDD data[22]. These data were obtained from autopsy iliac crest samples from a group of healthy individuals comprising 7 men and 18 women of a larger age range from 37 to 95 years (68.7±19.0 years; mean±SD)[22]. Bone matrix mineralization was shown to be relatively constant in healthy adults and independent of biological factors including sex and ethnicity[22-24]. Therefore, our reference BMDD are considered as "general" reference data for comparison with different patient groups.

Additionally, the mineral content of the bone tissue located between the tetracycline labels (CaYoung) was measured separately for information on the speed of matrix mineralization at the bone forming site. For this purpose, qBEI was combined with confocal laser scanning microscopy (CLSM-Leica TCS SP5, Leica Microsystems CMS GmbH, Wetzlar, Germany) (Figure 1C, D, E) to visualize the double labels. Laser light with a wavelength of 405 nm for fluorescence excitation and a 20x object lens (pixel resolution
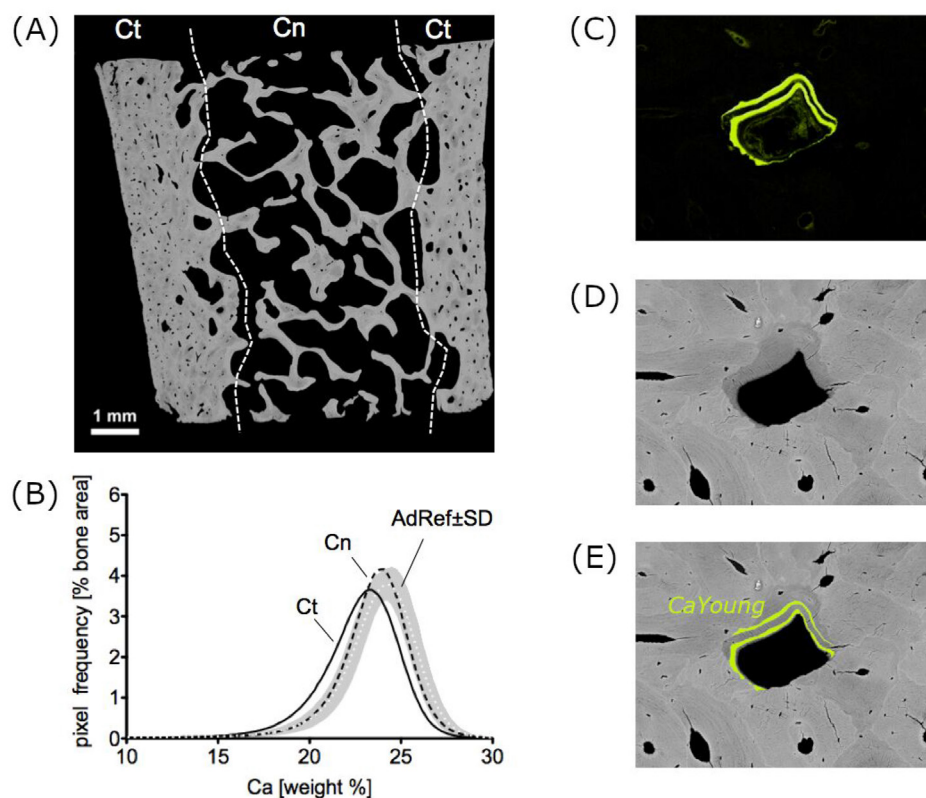
Figure 1. (A) qBEI image of the entire sectioned area from a transiliac T2DM bone biopsy sample. Noteworthy, the typically elevated trabecular bone volume is visible (BV/TV=29%, versus 21% of normal). Cn=cancellous and Ct=cortical compartment. (B) shows the corresponding BMDD curves. AdRef±SD=cancellous reference BMDD of adult healthy individuals (grey band indicates ±1 SD). (C) CLSM image of an osteon with tetracycline double labels, (D) subsequent identification of the identical osteon in qBEI (identical bone surface) and (E) overlay of qBEI and CLSM images.

0.76 μm) was used. For the exact matching of the qBEI with the CLSM images the focal plane of the CLSM was adjusted close to the bone sample surface by using the reflected light signal as described previously[25]. qBEI images from bone areas with double labels were subsequently acquired at the SEM at high magnification (200x nominal magnification, corresponding to a pixel resolution of 0.57 μm). The sites of the fluorescence labels were overlaid exactly onto the qBEI images by matching the CLSM images with the qBEI images using GIMP (GNU image manipulation program version 2.10.14). Then the mean calcium concentration between the double labels (CaYoung) could be analyzed for each sample using ImageJ software (version 1.50f; NIH, Bethesda, MD, USA)[26]. Per each of the 23 samples containing double labels, on average a bone area of about 32000 μm² was analyzed for CaYoung (3 samples were lacking double labels).

In order to analyze the 2D characteristics of osteocyte lacunae sections (OLS), a series of qBEI images was acquired with 130x nominal magnification corresponding to a pixel resolution of 0.88μm from the cancellous and cortical compartments. In each biopsy sample, on average n=1254

and n=807 OLS were recorded respectively per cortical and cancellous compartment and analyzed separately. The qBEI images were transformed to binary images using a threshold based on a fixed grey level (5.2 wt% Ca) (for details see previous work[19]). These binary images were analyzed for OLS based on a custom-made macro in ImageJ (version 1.50f; NIH, Bethesda, MD, USA)[26]. The size of the analyzed OLS was constrained to sizes between 5 μm² and 80 μm². Five parameters were calculated: (i) the OLS-density, which is the number of OLS per bone area; (ii) the OLS-porosity, which is the total OLS area normalized to bone area; (iii) the mean OLS-area; (iv) the mean OLS-perimeter; and (v) the OLS-aspect ratio, which is the mean ratio of the major and the minor axis of a fitted ellipse to each OLS. OLS-aspect ratio=1 indicates a perfect circle, increasing values indicate increasingly elongated shapes of the OLS. OLS-aspect ratio values >10 were excluded from the analysis.

The control group which was chosen for comparison with the patients' OLS-characteristics comprised n=9 healthy premenopausal women with an average age of 49.8±2.2 years (mean±SD, range 46.8 to 52.6 years). These represent

Table 1. Patients' characteristics and histomorphometric dynamic indices of bone formation.

	Patients with T2DM (n=26)
Age (yrs)	41.5 [38.0; 45.3]
Duration of T2DM (yrs)	8.0 [6.0; 13.3]
Body mass index	32.4 [28.9; 34.5]
Chronic complications (nb. of pat.)	9
Vertebral fractures[1] (nb. of pat.)	3
HbA1c (%)	7.6 [6.2; 9.6]
HbA1c <7% / >7% (nb. of pat.)	10 / 16
Medication	
Metformin (nb. of pat.)	23
SGLT2 (nb. of pat.)	3
Insulin (nb. of pat.)	13
Serum parameters	
FBG (mg/dL)	148.0 [103.8; 235.8]
Calcium (mg/dL)	9.2 [8.9; 9.5]
Phosphate	3.6 [3.3; 4.0]
Intact PTH (pg/dL)	44.9 [36.6; 60.8]
Pentosidine (pmol/mL)	3596 [1501; 4541]
Histomorphometric parameters	
BV/TV (%)	29.0 [23.0; 31.0][2]
MS/BS (%)	1.4 [0.5; 4.0][3]
MAR (µm/day)	0.61 [0.30; 0.71][3]
BFR/BS (µm^3·µm^{-2}·d^{-1})	0.007 [0.002; 0.023][3]

Data are median (25th, 75th percentiles).
[1]diagnosis was based on lateral X-ray of the thoracic and lumbar spine (from T4 to L4) by the method described by Genant[44].
[2]BV/TV of both subgroups with good (HbA1c<7%) or poor glycemic control (HbA1c>7%) are significantly higher compared to BV/TV of control group from previous publication[20].
[3]No comparison of dynamic indices with control group available from previous publication[20], however dynamic indices do not differ to premenopausal OLS-control group of the current work (all p>0.05).
Abbreviations: HbA1c = glycated; hemoglobin; SGLT2 = type 2 sodium-glucose cotransporter inhibitors; FBG = fasting blood glucose; PTH = parathyroid hormone

a subgroup of a study cohort reported previously[27] in whom a transiliac bone biopsy was performed. This age-matched control group of women was used for comparison with the patients group as the OLS-characteristics were reported to be age- and sex-dependent[28].

Statistical Analysis

Statistical analysis was performed using SigmaStat for Windows Version 4.0 (SPSS Inc.). Comparison of the patients' BMDD and OLS with reference data and comparison among the reported subgroups defined by clinical characteristics (HbA1c, etc.) was based on t-test or rank sum test (latter for non-normally distributed data). Correlation analysis was performed using Pearson product moment or Spearman rank order correlation (latter for non-normally distributed data). Intra-individual differences between cortical and cancellous compartments were studied based on paired t-test or Wilcoxon signed rank test (latter for non-normally distributed data). Two-sided p<0.05 was considered for significance.

Results

Clinical characteristics from the study cohort with T2DM are shown in Table 1.

No significant differences in BMDD between T2DM patients and BMDD reference cohort

Neither cancellous nor cortical BMDD-parameters from the patients were significant different from reference BMDD (Table 2). Further, no significant differences were found for cancellous or cortical BMDD between the subgroups defined by HbA1C (<7% versus >7%, data see Table 2), by chronic complications (with [n=9] versus without [n=17], data shown in Supplementary Table 1), by history of vertebral fractures (with [n=3] versus without [n=23], data shown in Supplementary Table 1), or by treatment with insulin (with [n=13] versus without [n=13], data shown in Supplementary Table 1).

When analyzing intra-individual differences between cancellous and cortical BMDD parameters we found highly significant differences in the T2DM patients as well as in in the reference cohort (Table 3). Compared to cortical bone, the cancellous compartment had for instance a 0.44 wt%Ca higher CaMean and a 0.20 wt%Ca lower CaWidth (all intra-individual differences within T2DM and reference cohort are listed in Table 3). These intra-individual differences in BMDD parameters between the compartments were similar between T2DM and reference (statistical comparison p>0.05). Furthermore, in T2DM CaYoung was found higher in the cancellous compartment: Ct.CaYoung 19.57 (18.94; 20.09) wt% and for Cn.CaYoung 20.19 (19.55; 20.73) wt% (median, [25th, 75th percentiles]) (pairwise comparison p<0.001). The mean intra-individual absolute difference of cancellous minus cortical CaYoung (ΔCaYoung) was 0.76 wt%Ca. Of note, no CaYoung data were available for the BMDD reference group, as these were post mortem samples lacking fluorescence labeling.

Despite the differences in BMDD parameters between cancellous and cortical bone compartments, CaMean was strongly correlated between these compartments in T2DM (R=0.85, p<0.001), similar to the reference cohort (R=0.77, p<0.001) shown in Figure 2A.

Strong correlations between BMDD parameters and histomorphometric indices of bone formation in T2DM patients

Correlation analysis was performed between BMDD parameters of T2DM patients and their previously measured, re-visited serum and histomorphometric data. BMDD

Table 2. Cohort BMDD median outcomes from patients with T2DM.

	Patients with diabetes mellitus type 2 (T2DM)			Adult reference[1]
	Total cohort (n=26)	**HbA1C<7% (n=10)**	**HbA1C>7% (n=16)**	**(n=25)**
Cn.CaMean (wt%)	23.25 (22.90; 23.89)	22.96 (22.79; 23.88)	23.43 (22.92; 23.95)	23.31 (23.12; 23.67)
Cn.CaPeak (wt%)	24.09 (23.74; 24.61)	23.92 (23.70; 24.52)	24.44 (23.74; 24.61)	24.09 (23.92; 24.44)
Cn.CaWidth (Δwt%)	3.81 (3.64; 4.33)	3.90 (3.64; 4.51)	3.73 (3.51; 4.16)	3.81 (3.81; 3.99)
Cn.CaLow (%B.Ar)	4.44 (3.33; 5.33)	4.26 (3.79; 5.81)	4.56 (3.12; 5.27)	4.61 (3.96; 5.48)
Cn.CaHigh (%B.Ar)	3.93 (2.60; 7.36)	3.04 (2.48; 7.41)	5.31 (2.51; 7.34)	3.99 (3.17; 5.56)
Ct.CaMean (wt%)	22.91 (22.44; 23.31)	22.82 (22.33; 23.49)	22.92 (22.49; 23.21)	23.04 (22.68; 23.24)
Ct.CaPeak (wt%)	23.74 (23.29; 24.02)	23.66 (23.09; 24.22)	23.79 (23.44; 23.98)	23.92 (23.66; 24.26)
Ct.CaWidth (Δwt%)	4.07 (3.73; 4.27)	3.99 (3.79; 4.55)	4.07 (3.66; 4.25)	4.07 (3.90; 4.25)
Ct.CaLow (%B.Ar)	5.41 (3.99; 6.60)	4.68 (3.68; 6.52)	5.66 (4.47; 6.66)	5.18 (4.80; 6.35)
Ct.CaHigh (%B.Ar)	3.04 (1.52; 4.61)	2.28 (1.42; 4.86)	3.42 (1.73; 4.51)	3.70 (2.31; 4.78)

[1]Adult reference group comprised healthy men and women aged 30 to 95 years[22].
Data are median (25th, 75th percentiles). Comparison to reference as well as subgroup comparison by HbA1c failed statistical significance.

Table 3. Intra-individual differences between cancellous and cortical compartments within patients with T2DM and within healthy individuals.

	Patients with diabetes		Healthy individuals from reference cohorts	
	Mean intra-individual difference Δ (Cn − Ct)	Significance (by pairwise comparison)	Mean intra-individual difference Δ (Cn − Ct)	Significance (by pairwise comparison)
CaMean (wt%)	0.44	<0.001	0.30	<0.001
CaPeak (wt%)	0.46	<0.001	0.25	0.002
CaWidth (Δwt%)	-0.20	0.002	-0.23	0.012
CaLow (%B.Ar)	-0.79	<0.001	-0.64	0.049
CaHigh (%B.Ar)	2.09	<0.001	0.87	0.010
CaYoung (wt%)	0.76	<0.001	n.a.[1]	n.a.
OLS-porosity (%)	-0.04	0.012	0.04	n.s.
OLS-density (nb/mm²)	-30.7	0.002	-3.6	n.s.
OLS-area (μm²)	1.04	n.s.	2.14	n.s.
OLS-perimeter (μm)	1.04	0.02	1.28	n.s.
OLS-aspect ratio	0.30	<0.001	0.26	n.s.

Mean intra-individual difference is the mean of the absolute differences from each individual of the study group.
Significance represents the p-value based on paired t-tests or Wilcoxon signed rank tests.
[1]n.a.=not available as iliac crest bone samples from reference cohort[22] are autopsy samples which are not tetracycline labeled.
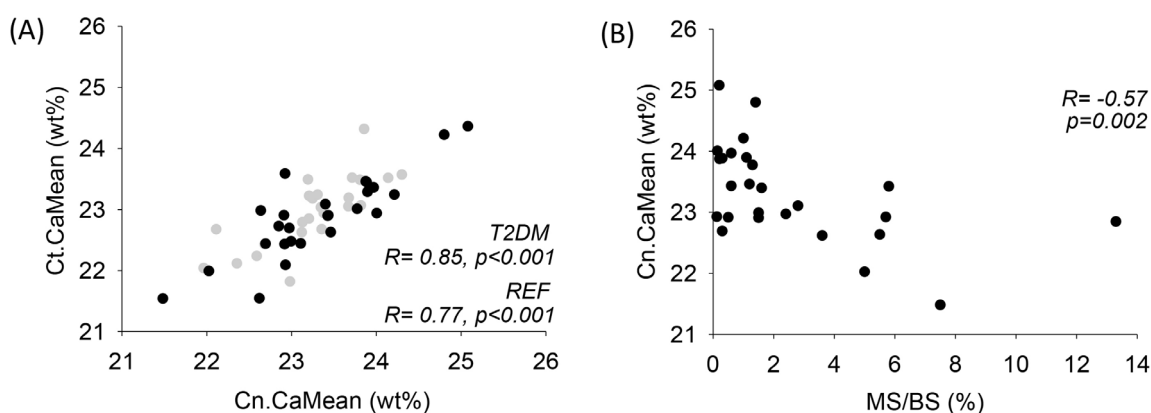

Figure 2. Correlation analyses. (A) Cancellous CaMean plotted versus cortical CaMean in T2DM (black symbols) and in the healthy reference cohort (light grey symbols). (B) Cancellous CaMean from patients with T2DM in relation to previously obtained mineralizing surface per bone surface (MS/BS). Correlation coefficients R and corresponding p-values are shown.
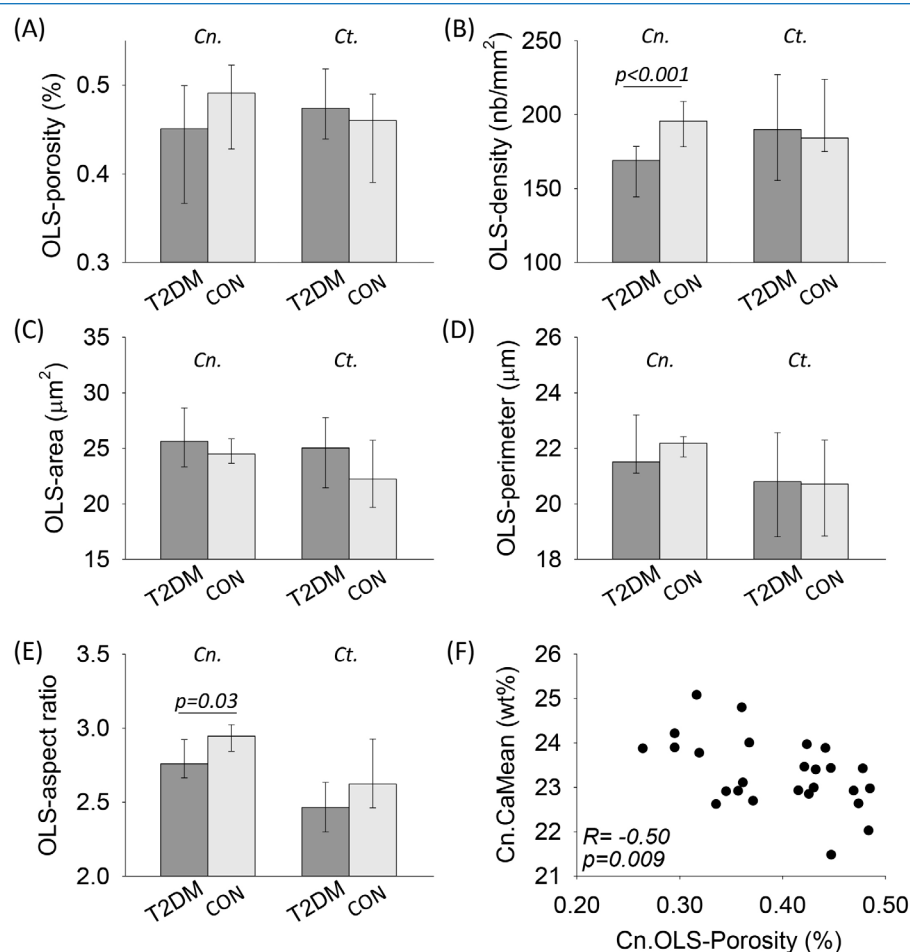
Figure 3. (A-E) OLS-characteristics from patients with T2DM (dark grey) and age- and sex-matched control (CON) samples (light grey) in cancellous (Cn.) and cortical (Ct.) (left and right columns respectively). Data show median (25th; 75th percentiles). (F) Cancellous CaMean in relation to cancellous OLS-porosity in samples from patients with T2DM.

outcomes were neither correlated with serum or urine parameters (including serum calcium, phosphate, alkaline phosphatase, fasting blood glucose (FBG), triglyceride, HDL, IGF-1, microalbumin, creatinine) (data not shown, all correlations p>0.05). Only, a weak correlation was found between Cn.CaWidth and iPTH (R=0.42, p=0.02). Statistically significant correlations were found between BMDD and histomorphometric indices of bone formation (Table 4, Figure 2B). These correlations indicated that the higher the rate of bone formation the lower was the overall degree of mineralization (CaMean and CaPeak), the higher was the heterogeneity of mineralization (CaWidth) and the higher was the percentage of bone areas with low mineralization (CaLow).

Lowered OLS-density in cancellous bone of T2DM patients

The analysis of OLS-characteristics revealed, that two of the ten OLS indices differed in T2DM from the healthy premenopausal control group (Figure 3A-E): Cancellous OLS-density was significantly lowered by 14% and OLS-AR by 6.4% (median comparison) in our patients. However, cortical OLS-characteristics were found within control reference range. Subgroups defined by clinical characteristics (HbA1c <7% versus >7%, with versus without chronic complications, with versus without fragility fractures) and treatment (with versus without insulin treatment) were compared and did not show any significant differences in OLS-characteristics (data shown in Supplementary Table 1). Noteworthy, we could also observe a significant negative relationship between cancellous OLS-porosity and Cn.CaMean in the T2DM group (Figure 3F).

Discussion

An important outcome of our study was that the BMDD from the patients with T2DM did not differ from our reference

Table 4. Correlation of cancellous BMDD and CaYoung with re-visited histomorphometric indices of bone formation in the T2DM study group.

	BFR/BS		
	MS/BS	MAR	BFR/BS
Cn.CaMean	R=-0.52, p=0.006	R= -0.42, p=0.03	R= -0.52, p=0.007
Cn.CaPeak	R= -0.46, p=0.02	n.s.	R= -0.45, p=0.02
Cn.CaWidth	R=0.64, p<0.001	R=0.42, p=0.03	R=0.65, p<0.001
Cn.CaLow	R=0.56, p=0.003	n.s.	R=0.56, p=0.003
Cn.CaHigh	n.s.	R= -0.40, p=0.04	n.s.
Cn.OLS-porosity	R=0.49, p<0.05	R=0.54, p=0.004	R=0.50, p=0.009
Cn.OLS-density	R=0.39, p<0.05	n.s.	n.s.
Cn.OLS-area	n.s.	n.s.	n.s.
Cn.OLS-perimeter	n.s.	n.s.	n.s.
Cn.OLS-aspect ratio	n.s.	n.s.	n.s.
Cn.CaYoung	n.s.	n.s.	n.s.

Data are PPM or SRO correlation coefficients and corresponding p-values. n.s. = not significant.
Histomorphometric indices of bone formation are re-visited outcomes from our previous study[20].

data. Noteworthy, the bone mineral density measured by DXA (which is reflecting a combination of both bone volume and mineral content[29]) in our patients was high[9] and exceeded on average the 75th percentile of the published healthy age- and sex-matched reference range[30]. The present observation of normal bone mineralization together with the previously reported increased bone volume[20] suggests that the high bone mineral density by DXA is caused by high bone volume rather than by increased bone material density.

One essential determinant for the BMDD (by qBEI) is the level of bone formation/turnover[18], which is clearly visible in the significant correlation of BMDD with histomorphometric indices of bone formation in the present patient cohort and observed generally[31]. The average level of histomorphometric bone formation (mineralizing surface per bone surface) from our patients[20] was relatively low but did not differ from that of our premenopausal healthy OLS-control group (statistical comparison p>0.05, data not shown) and was of the same range reported previously for another healthy premenopausal cohort[32]. This is in contrast to the reduced levels of serum bone turnover markers in type-2 diabetes mellitus reported by others[3,33,34] but might be due to the young age and reflect a positive influence of treatment in our cohort. Noteworthy, half of our patients were treated with insulin, which is known to be osteoanabolic[1,35] and might have pushed bone formation towards normal level in most severely affected patients. We hypothesize that without insulin treatment these severely affected patients would have had reduced bone turnover. However, this assumption remains speculative as no biopsy before insulin treatment was available in these patients.

Moreover, we characterized the porosity, density and shape parameters of the osteocyte lacunae sections (OLS) based on the qBEI images. It has to be noted that mineralized osteocyte lacunae which were reported as a marker for pathologies and/or old tissue age[36] as well as the viability of the osteocytes were not taken into account by this analysis.

We observed a highly significant decrease in OLS density in cancellous bone, which might be more affected than cortical bone as it is remodeled at a faster rate generally[39]. Our findings suggest that a lower number of cells got embedded into the bone material which in turn might point towards an altered differentiation of osteoblasts to osteocytes in T2DM. It might also be possible that the chronic hyperglycemia and subsequent AGEs accumulation have a direct effect on osteocytes[16] and their function/mechanosensitivity[37]. *In vitro* studies showed that MLO-Y4 cells' proliferation and viability decreased under high glucose condition[38].

Furthermore, we could not observe any significant BMDD or OLS differences between subgroups defined by parameters which characterized the severity of T2DM. These subgroups were good versus poor glycemic control and with versus without chronic complications. Given this lack of difference and the similarity of the BMDD curve with the reference curve, we conclude that other factors than bone matrix mineralization are contributing to the decreased bone quality in our group of patients. Such factors might be accumulation of AGEs[9] or an increased cortical porosity at fracture relevant sites (as described in other cohorts with T2DM[40,41]).

Due to previously reported differences in AGEs between cortical and cancellous bone in our patients with T2DM[9], we studied potential differences in bone matrix mineralization between these compartments. In line with previous observations[31], we found cancellous and cortical BMDD significantly correlated in the iliac crest. In both, patients and reference cohort, mineralization was lower in cortical bone than that of cancellous bone, which was unexpected given the generally lower intra-cortical bone turnover[39].

For further clarification, we measured "CaYoung" which is defined as the mean mineral content exclusively between the tetracycline double labels[25] and thus provides information on the speed of mineral accumulation during

the first weeks within the newly formed bone. In general, when bone is formed during remodeling, the organic matrix starts to mineralize after about two weeks of maturation[39], followed by a primary fast and a secondary slower mineral accumulation. From a recent work, we know that the age of the bone tissue between the double labels represents the mineral content which is achieved after the completion of primary mineralization[25]. Thus, the lower CaYoung value found in the cortex of the ilium suggests that the early phase of mineralization is slower in the cortex which is in line with the higher nano-porosity (a surrogate for bone material water content) at bone formation sites in the cortex as measured by Raman microspectroscopy in our patients[9]. This suggests that a lower amount of free water in the bone matrix was replaced by mineral crystals during the mineralization process in cortical bone. Furthermore, it is likely that also the level of late secondary mineralization is somewhat lower in the cortex as a significantly smaller percentage of bone area has a mineral content beyond 26.86 wt%Ca, i.e., CaHigh is lower in the cortex compared to cancellous bone. These data possibly reflect some general differences in mineral accumulation between cortical and trabecular bone, which might arise from principally differing mechanism of matrix mineralization and/or from unequal access to calcium ions for mineralization[43] given the differences in bone-marrow-surface.

Our work has some limitations. First, no tetracycline labeling was available in our BMDD reference cohort because the latter originates from autopsy samples from healthy donors[22]. Second, for definite conclusions about the mineralization kinetics, more than one set of tetracycline double labels would be essential which is generally difficult to achieve in patients. Third, it will be important to expand our control cohort for OLS-characteristics. The BMDD reference cohort could not be used for this purpose as it comprises individuals of a large age range up to 95 years. As a consequence, to the observed age-dependency of OLS-characteristics[28] we confined reference data to a healthy premenopausal control group. Another limitation of our work is that no further subgroups by treatment could be studied due to the limited sample size of these subgroups. In this context, for instance the effect of SGTL2 which is discussed to have adverse effects on bone health, would have been of interest. However, the BMDD data of the three patients who were treated with SGTL2 lie perfectly within the trend of the entire study group. For OLS-characteristics, one SGTL2 treated patient had the highest cancellous OLS-porosity, highest cortical OLS-area, and highest OLS-perimeter among all T2DM patients. Thus, a larger group of treated patients would be needed to clarify any potential effect of SGTL2 on bone biopsy characteristics. Finally, our study cohort was relatively young and thus our findings might not be extrapolated to the more typical older populations with T2DM.

For conclusion, we detected only minor differences in bone material in T2DM versus reference. The generally normal bone matrix mineralization is in line with the relatively normal level of histomorphometric bone formation in our patients with T2DM. This might be due to the relatively young age of the patients or might also indicate a successful medication. The observed differences between cancellous and cortical BMDD in our patients might provide new insights into differences in mineralization kinetics in these compartments in general.

References

1. Pietschmann P, Patsch JM, Schernthaner G. Diabetes and bone. Horm Metab Res 2010;42:763-8.
2. Starup-Linde J, Hygum K, Harsløf T, Langdahl B. Type 1 Diabetes and Bone Fragility: Links and Risks. Diabetes Metab Syndr Obes 2019;12:2539-2547.
3. Compston J. Type 2 diabetes mellitus and bone. J Intern Med 2018;283:140-153.
4. Karim L, Bouxsein ML. Effect of type 2 diabetes-related non-enzymatic glycation on bone biomechanical properties. Bone 2016;82:21-7.
5. Yamamoto M, Sugimoto T. Advanced Glycation End Products, Diabetes, and Bone Strength. Curr Osteoporos Rep 2016;14:320-326.
6. Furst JR, Bandeira LC, Fan WW, Agarwal S, Nishiyama KK, McMahon DJ, Dworakowski E, Jiang H, Silverberg SJ, Rubin MR. Advanced Glycation Endproducts and Bone Material Strength in Type 2 Diabetes. J Clin Endocrinol Metab 2016;101:2502-10.
7. Napoli N, Chandran M, Pierroz DD, Abrahamsen B, Schwartz AV, Ferrari SL; IOF Bone and Diabetes Working Group. Mechanisms of diabetes mellitus-induced bone fragility. Nat Rev Endocrinol 2017;13:208-219.

8. Picke AK, Campbell G, Napoli N, Hofbauer LC, Rauner M. Update on the impact of type 2 diabetes mellitus on bone metabolism and material properties. Endocr Connect 2019;8:R55-R70.

9. Rokidi S, Andrade VFC, Borba V, Shane E, Cohen A, Zwerina J, Paschalis EP, Moreira CA. Bone tissue material composition is compromised in premenopausal women with Type 2 diabetes. Bone 2020;141:115634.

10. Bala Y, Depalle B, Douillard T, Meille S, Clément P, Follet H, Chevalier J, Boivin G. Respective roles of organic and mineral components of human cortical bone matrix in micromechanical behavior: an instrumented indentation study. J Mech Behav Biomed Mater 2011;4:1473-82.

11. Lekkala S, Taylor EA, Hunt HB, Donnelly E. Effects of Diabetes on Bone Material Properties. Curr Osteoporos Rep 2019;17:455-464.

12. Sihota P, Yadav RN, Dhaliwal R, Bose JC, Dhiman V, Neradi D, Karn S, Sharma S, Aggarwal S, Goni VG, Mehandia V, Vashishth D, Bhadada SK, Kumar N. Investigation of mechanical, material and compositional determinants of human trabecular bone quality in type 2 diabetes. J Clin Endocrinol Metab 2021:dgab027. Online ahead of print.

13. Karim L, Moulton J, Van Vliet M, Velie K, Robbins A, Malekipour F, Abdeen A, Ayres D, Bouxsein ML. Bone microarchitecture, biomechanical properties, and advanced glycation end-products in the proximal femur of adults with type 2 diabetes. Bone 2018;114:32-39.

14. Pritchard JM, Papaioannou A, Tomowich C, Giangregorio LM, Atkinson SA, Beattie KA, et al. Bone mineralization is elevated and less heterogeneous in adults with type 2 diabetes and osteoarthritis compared to controls with osteoarthritis alone. Bone 2013;54:76–82.

15. Hunt HB, Torres AM, Palomino PM, Marty E, Saiyed R, Cohn M, Jo J, Warner S, Sroga GE, King KB, Lane JM, Vashishth D, Hernandez CJ, Donnelly E. Altered Tissue Composition, Microarchitecture, and Mechanical Performance in Cancellous Bone From Men With Type 2 Diabetes Mellitus. J Bone Miner Res 2019;34:1191-1206.

16. Piccoli A, Cannata F, Strollo R, Pedone C, Leanza G, Russo F, Greto V, Isgrò C, Quattrocchi CC, Massaroni C, Silvestri S, Vadalà G, Bisogno T, Denaro V, Pozzilli P, Tang SY, Silva MJ, Conte C, Papalia R, Maccarrone M, Napoli N. Sclerostin Regulation, Microarchitecture, and Advanced Glycation End-Products in the Bone of Elderly Women With Type 2 Diabetes. J Bone Miner Res 2020; 35:2415-2422.

17. Hunt HB, Miller NA, Hemmerling KJ, Koga M, Lopez KA, Taylor EA, Sellmeyer DE, Moseley KF, Donnelly E. Bone Tissue Composition in Postmenopausal Women Varies With Glycemic Control From Normal Glucose Tolerance to Type 2 Diabetes Mellitus. J Bone Miner Res 2021; 36:334-346.

18. Roschger P, Paschalis EP, Fratzl P, Klaushofer K. Bone mineralization density distribution in health and disease. Bone 2008;42:456-66.

19. Blouin S, Fratzl-Zelman N, Glorieux FH, Roschger P, Klaushofer K, Marini JC, Rauch F. Hypermineralization and High Osteocyte Lacunar Density in Osteogenesis Imperfecta Type V Bone Indicate Exuberant Primary Bone Formation. J Bone Miner Res 2017:32(9):1884-1892.

20. Andrade VFC, Chula DC, Sabbag FP, Cavalheiro DDDS, Bavia L, Ambrósio AR, da Costa CRV, Dos Reis LM, Borba VZC, Moreira CA. Bone Histomorphometry in Young Patients With Type 2 Diabetes is Affected by Disease Control and Chronic Complications. J Clin Endocrinol Metab 2020:105:dgz070.

21. Gavin J, Alberti K, Davidson M, DeFronzo R, Drash A, Gabbe S, Genuth S, Harris M, Kahn R, Keen H, Knowler W, Lebovitz H, Maclaren N, Palmer J, Raskin P, Rizza R, Stern M. Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care 2003;26(suppl 1): s5-s20.

22. Hartmann MA, Blouin S, Misof BM, Fratzl-Zelman N, Roschger P, Berzlanovich A, Gruber GM, Brugger PC, Zwerina J, Fratzl P. Quantitative Backscattered Electron Imaging of bone using a thermionic or a field emission electron source. Calcif Tissue Int 2021;109(2):190-202.

23. Roschger P, Gupta HS, Berzlanovich A, Ittner G, Dempster DW, Fratzl P, Cosman F, Parisien M, Lindsay R, Nieves JW, Klaushofer K. Constant mineralization density distribution in cancellous human bone. Bone 2003;32:316-323.

24. Boivin G and Meunier PJ. The degree of mineralization of bone tissue measured by computerized quantitative contact microradiography. Calcif Tissue Int 2002; 70:503-511.

25. Misof BM, Roschger P, Zhou H, Nieves JW, Bostrom M, Cosman F, Lindsay R, Klaushofer K, Dempster DW. No evidence for alteration in early secondary mineralization by either alendronate, teriparatide or combination of both in transiliac bone biopsy samples from postmenopausal osteoporotic patients. Bone Rep 2020;12:100253. eCollection 2020 Jun.

26. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671–5.

27. Misof BM, Roschger P, Blouin S, Recker R, Klaushofer K. Bone matrix mineralization is preserved during early perimenopausal stage in healthy women: a paired biopsy study. Osteoporos Int 2016;27:1795-803.

28. Bach-Gansmo FL, Brüel A, Jensen MV, Ebbesen EN, Birkedal H, Thomsen JS. Osteocyte lacunar properties and cortical microstructure in human iliac crest as a function of age and sex. Bone 2016;91:11-9.

29. Fratzl P, Roschger P, Fratzl-Zelman N, Paschalis EP, Phipps R, Klaushofer K. Evidence that treatment with risedronate in women with postmenopausal osteoporosis affects bone mineralization and bone volume. Calcif Tissue Int 2007;81:73-80.

30. Alvarenga JC, Fuller H, Pasoto SG, Pereira RM. Age-related reference curves of volumetric bone density,

structure, and biomechanical parameters adjusted for weight and height in a population of healthy women: an HR-pQCT study. Osteoporos Int 2017;28:1335-1346.

31. Misof BM, Dempster DW, Zhou H, Roschger P, Fratzl-Zelman N, Fratzl P, Silverberg SJ, Shane E, Cohen A, Stein E, Nickolas TL, Recker RR, Lappe J, Bilezikian JP, Klaushofer K. Relationship of bone mineralization density distribution (BMDD) in cortical and cancellous bone within the iliac crest of healthy premenopausal women. Calcif Tissue Int 2014;95:332-9.

32. Misof BM, Gamsjaeger S, Cohen A, Hofstetter B, Roschger P, Stein E, Nickolas TL, Rogers HF, Dempster D, Zhou H, Recker R, Lappe J, McMahon D, Paschalis EP, Fratzl P, Shane E, Klaushofer K. Bone material properties in premenopausal women with idiopathic osteoporosis. J Bone Miner Res 2012;27:2551-61.

33. Purnamasari D, Puspitasari MD, Setiyohadi B, Nugroho P, Isbagio H. Low bone turnover in premenopausal women with type 2 diabetes mellitus as an early process of diabetes-associated bone alterations: a cross-sectional study. BMC Endocr Disord 2017;17:72.

34. Hygum K, Starup-Linde J, Harsløf T, Vestergaard P, Langdahl BL. Eur J Endocrinol. MECHANISMS IN ENDOCRINOLOGY: Diabetes mellitus, a state of low bone turnover - a systematic review and meta-analysis 2017;176:R137-R157.

35. Murray CE, Coleman CM. Impact of Diabetes Mellitus on Bone Health. Int J Mol Sci 2019;20:4873.

36. Milovanovic P, Busse B. Phenomenon of osteocyte lacunar mineralization: indicator of former osteocyte death and a novel marker of impaired bone quality? Endocr Connect 2020;9:R70-80.

37. Gortázar AR, Ardura JA. Osteocytes and Diabetes: Altered Function of Diabetic Osteocytes. Curr Osteoporos Rep 2020;18:796-802.

38. Sun T, Yan Z, Cai J, Shao X, Wang D, Ding Y, Feng Y, Yang J, Luo E, Feng X, Jing D. Effects of mechanical vibration on cell morphology, proliferation, apoptosis, and cytokine expression/secretion in osteocyte-like MLO-Y4 cells exposed to high glucose. Cell Biol Int 2019 Aug 26. Online ahead of print.

39. Qiu S, Divine G, Warner E, Rao SD. Reference Intervals for Bone Histomorphometric Measurements Based on Data from Healthy Premenopausal Women. Calcif Tissue Int 2020;107:543-550.

40. Patsch JM, Burghardt AJ, Yap SP, Baum T, Schwartz AV, Joseph GB, Link TM. Increased cortical porosity in type 2 diabetic postmenopausal women with fragility fractures. J Bone Miner Res 2013;28:313-24.

41. Samakkarnthai P, Sfeir JG, Atkinson EJ, Achenbach SJ, Wennberg PW, Dyck PJ, Tweed AJ, Volkman TL, Amin S, Farr JN, Vella A, Drake MT, Khosla S. Determinants of Bone Material Strength and Cortical Porosity in Patients with Type 2 Diabetes Mellitus. J Clin Endocrinol Metab 2020;105:e3718-29.

42. Lerebours C, Weinkamer R, Roschger A, Buenzli PR. Mineral density differences between femoral cortical bone and trabecular bone are not explained by turnover rate alone. Bone Rep 2020;13:100731. eCollection 2020 Dec.

43. Roschger A, Wagermaier W, Gamsjaeger S, Hassler N, Schmidt I, Blouin S, Berzlanovich A, Gruber GM, Weinkamer R, Roschger P, Paschalis EP, Klaushofer K, Fratzl P. Newly formed and remodeled human bone exhibits differences in the mineralization process. Acta Biomater 2020;104:221-230.

44. Genant HK. Assessment of vertebral fractures in osteoporosis research. J Rheumatol 1997;24:1212-4.

Supplementary Table 1. BMDD and OLS comparison of subgroups defined by clinical characteristics or treatment.

BMDD-OUTCOMES			
	With insulin tx (n=13)	Without insulin tx (n=13)	p-value
Cn.CaMean [wt%Ca]	23.48 (0.66)	23.16 (0.91)	0.304
Cn.CaPeak [wt%Ca]	24.30 (0.58)	23.98 (0.74)	0.232
Cn.CaWidth [Δwt%Ca]	3.80 (0.36)	3.95 (0.47)	0.383
Cn.CaLow [%B.Ar]	4.33 (1.04)	4.82 (1.75)	0.397
Cn.CaHigh [%B.Ar]	5.03 (2.76; 7.65)	3.06 (1.93; 6.71)	0.305
	With CC (n=9)	Without CC (n=17)	p-value
Cn.CaMean [wt%Ca]	23.54 (0.51)	23.20 (0.90)	0.308
Cn.CaPeak [wt%Ca]	24.34 (0.43)	24.04 (0.76)	0.286
Cn.CaWidth [Δwt%Ca]	3.72 (0.37)	3.96 (0.43)	0.171
Cn.CaLow [%B.Ar]	4.04 (0.95)	4.86 (1.59)	0.171
Cn.CaHigh [%B.Ar]	5.59 (3.47; 7.42)	3.06 (2.32; 7.39)	0.388
	With fragility fx (n=3)	Without fragility fx (n=23)	p-value
Cn.CaMean [wt%Ca]	23.19 (0.68)	23.34 (0.82)	0.778
Cn.CaPeak [wt%Ca]	23.92 (0.46)	24.17 (0.70)	0.545
Cn.CaWidth [Δwt%Ca]	3.64 (0.17)	3.90 (0.43)	0.314
Cn.CaLow [%B.Ar]	3.43 (1.00)	4.18 (1.18)	0.310
Cn.CaHigh [%B.Ar]	17.60 (8.83)	24.51 (12.78)	0.377
OLS-CHARACTERISTICS			
	HbA1c>7% (n=16)	HbA1c<7% (n=10)	p-value
OLS-porosity (%)	0.43 (0.09)	0.43 (0.08)	0.846
OLS-density (nb./mm^2)	163.7 (17.5)	168.0 (26.6)	0.622
OLS-area (µm^2)	26.3 (3.7)	25.4 (3.0)	0.530
OLS-perimeter (µm)	22.1 (1.6)	21.9 (1.0)	0.728
OLS-AR	2.8 (0.2)	2.8 (0.2)	0.419
	With insulin tx (n=13)	Without insulin tx (n=13)	p-value
OLS-porosity (%)	0.40 (0.06)	0.39 (0.07)	0.725
OLS-density (nb./mm^2)	160.1 (15.3)	164.0 (24.7)	0.631
OLS-area (µm^2)	24.9 (2.5)	23.8 (2.3)	0.244
OLS-perimeter (µm)	21.6 (1.1)	21.4 (1.1)	0.658
OLS-AR	2.7 (0.2)	2.9 (0.2)	0.069
	With CC (n=9)	Without CC (n=17)	p-value
OLS-porosity (%)	0.38 (0.05)	0.40 (0.07)	0.469
OLS-density (nb./mm^2)	155.3 (11.0)	165.6 (23.3)	0.223
OLS-area (µm^2)	24.5 (2.3)	24.2 (2.5)	0.784
OLS-perimeter (µm)	21.4 (1.1)	21.5 (1.1)	0.861
OLS-AR	2.7 (0.1)	2.8 (0.2)	0.307
	With fragility fx (n=3)	Without fragility fx (n=23)	p-value
OLS-porosity (%)	0.38 (0.04)	0.40 (0.07)	0.770
OLS-density (nb./mm^2)	158.5 (14.7)	162.5 (21.1)	0.754
OLS-area (µm^2)	24.2 (1.1)	24.3 (2.5)	0.956
OLS-perimeter (µm)	20.9 (0.4)	21.5 (1.1)	0.375
OLS-AR	2.63 (0.08)	2.8 (0.2)	0.093

Abbreviations: insulin tx=insulin treatment; CC=chronic complications including retinopathy and diabetic nephropathy; fragility fx=fragility fractures.
Data are mean (SD) and p-value from corresponding t-test or median (25th; 75th percentiles) and p-value from corresponding Mann-Whitney rank sum test (latter for non-normally distributed data). None of these comparisons is statistically different.