Osteocyte lacunar properties and cortical microstructure in human iliac crest as a function of age and sex

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A supplemental Table 1 and a supplemental Figure 1 has been included.

Abstract

Osteocytes are suggested to play a central role in bone remodeling. Evaluation of iliac crest biopsies is a standard procedure for evaluating bone conditions in the clinical setting. Despite the widespread use of such biopsies, little is known about the population of osteocytes in the iliac crest from normal individuals. Contradicting results have been reported on osteocyte lacunar properties in human bone. Hence, a solid understanding of the osteocyte population in healthy bone and the effect of age and sex is needed as good reference data are lacking. Furthermore, the role of cortical bone in bone quality has recently been suggested to be more important than previously realized. Therefore, the present study assesses osteocyte lacunar properties and cortical microstructure of the iliac crest as a function of age and sex. A total of 88 iliac crest bone samples from healthy individuals (46 women, aged 18.5–96.4 years and 42 men, aged 22.6–94.6 years) with an even age-distribution were examined using synchrotron radiation μ CT and in house μ CT, with more than 5×10^6 osteocyte lacunae measured and analyzed. The study revealed that osteocyte lacunar volumes were unaffected by both age and sex. Osteocyte lacunar density did not differ between women and men, and only showed a significant decrease with age when pooling data from both sexes. Cortical porosity and Haversian canal density increased while cortical thickness decreased with age, with cortical thinning dominating the age-related cortical bone loss. None of the cortical microstructural parameters showed any sex dependency. Only weak links between osteocyte lacunar properties and cortical microstructural properties in iliac crest bone were found. Interestingly, the Haversian canal diameters were significantly but weakly negatively correlated with osteocyte lacunar volumes.

Keywords: Osteocytes; Osteocyte lacunae; Iliac crest; Cortical bone; Age; Sex; Nano-CT

1. Introduction

The processes behind the age-related effects on bone are not fully understood, and with the increasing lifespan of the human population, this question is becoming increasingly relevant. Traditionally, age-related changes in bone have been considered more pronounced in women than in men, and therefore many studies have been conducted in women only. However, it remains unclear whether the age-related changes are dependent on sex or not, as there are several studies showing little or no difference between the sexes [1–4]. Most studies concerning aging of the normal skeleton have been performed in trabecular bone, whereas aging of cortical bone has been much less explored. This is surprising since cortical bone comprise approximately 80% of the total bone mass in the skeleton [5]. Moreover, cortical geometry and porosity has been linked to stiffness and strength of human bone [6, 7], and loss of cortical bone has been related to proximal femur fractures [8]. Increasing the understanding of the cortical bone compartment therefore seems necessary in order to reveal its role in bone quality, as also pointed out by other researchers [9, 10].

Recently, osteocytes have gained increasing attention, as it is becoming clear that they play a central role in the control of bone remodeling [11]. Osteocytes are by far the most abundant cells in bone, the number of osteocytes is estimated as ten times that of osteoblasts and thousand times that of osteoclasts [12]. Osteocytes are located in osteocyte lacunae throughout the bone matrix and are connected to other osteocytes via cellular processes running through canaliculi in the bone matrix. Multiple different roles have been suggested for the osteocytes including acting as mechanosensors [11, 13], removing perilacunar bone matrix (osteocytic osteolysis) [11, 14, 15], contributing to the regulation of osteoblasts through production of sclerostin [16], and contributing to recruiting osteoclasts through production of RANKL [17–19].

Different studies of human bone have shown varying results for osteocyte lacunar properties such as osteocyte lacunar volume, and it remains unknown whether osteocyte lacunar volume

differs between women and men [20–25]. Additionally, it is becoming increasingly clear that large ranges and variations in osteocyte lacunar properties exist in normal individuals, even within the same anatomical site, hence making studies of large sample sizes necessary [20–23, 26]. As a consequence, it is essential to determine the osteocyte lacunar properties in normal bone, and to investigate whether these properties change with age and whether they differ between women and men so that reference values can be ascertained.

The osteocyte lacunar geometric properties have previously been assessed using 2D techniques [27–29]. Since osteocyte lacunae are strongly anisotropic in shape, 2D methods are afflicted by potential sample sectioning bias. Therefore, 3D methods such as synchrotron based CT or confocal microscopy [30] are more appropriate [31]. Yet even with these tools, practical limitations arise from the compromise that must be made between resolution, investigated volume, and the number of individuals investigated. CT methods using synchrotrons have advanced tremendously and can now deliver sub-100 nm resolution [32–34], but studies in the literature continue to remain affected by either restricted volumes investigated, limited resolution, or a limited number of subjects.

Indeed, only a few studies containing many subjects have been conducted on osteocyte lacunae in human bone. Thus, osteocyte lacunae in cortical bone from the proximal femoral shaft of women aged 20–86 years were studied using synchrotron radiation (SR) μ CT [23]. When dividing the subjects into groups of women younger or older than 50 years, the osteocyte lacunar volumes were approximately 30% larger in the younger than in the older women, but no significant change in osteocyte lacunar density were found with age. In contrast, osteocyte lacunar density was reported to decrease with age in trabecular bone of transiliac crest biopsies [28]. No significant differences were found in size or shape of osteocyte lacunae in women with and without osteoporotic fractures in trabecular bone from femoral heads using confocal microscopy, but a large range of size and shapes were noted for each group [22]. Vashishth et al. found different effects of aging on osteocyte

lacunar density in human vertebral cancellous bone from women and men using histomorphometry [27]. They also reported larger coefficient of variation in men than in women with age, and explained this with decreased bone turnover in men with age, hence increasing heterogeneity. The lack of a clear picture of the sex-dependence and age development of osteocyte lacunar properties therefore calls for a comprehensive study covering both sexes and a large age-span.

The mechanical properties of cortical bone is related to its porosity [35–37] and thickness [38]. Likewise, increased cortical porosity at the iliac crest [39] as well as other skeletal sites has been linked to increased fracture risk with age [40, 41]. Furthermore, the cortical thickness is reduced with age [42–44]. Most previous studies of the age-related changes in cortical morphology in normal individuals has been conducted using conventional 2D histomorphometry, whereas studies of the age-related changes of the cortical morphology in normal individuals using μ CT based 3D methods has been almost absent.

In contrast to e.g. the vertebral body, a substantial cortical shell with Haversian remodeling is found at the iliac crest. Moreover, iliac crest bone biopsies remains a valuable, widely used, and well-established clinical and research tool for studying the etiology, pathogenesis, and treatment of metabolic bone disorders [45]. Therefore, a solid understanding of the properties and ageing in healthy cortical bone at this skeletal site using 3D methods is paramount. Consequently, the aim of the present study was to investigate age-related changes of human iliac crest cortical bone morphology in normal women and men on different length scales and to determine whether the changes in microstructural parameters are accompanied by changes in osteocyte lacunar properties.

2. Materials and methods

2.1. Bone samples

Iliac crest bone samples from 46 women aged 18.5–96.4 years and 42 men aged 22.6–94.6 years with an even age-distribution were included in the study. Individuals with known cancer, metabolic diseases, severe liver or kidney diseases, medication affecting bone metabolism, or periods of more than 2 weeks of immobilization prior to death were excluded from the study. The present study is a subset of a larger study called the “Danish in Vitro Bone Study” (DAVIBO) [2, 4, 46–48]. The collection of the material was approved by the local ethical committee.

At autopsy, approximately 5 cm × 5 cm iliac crest specimens were extracted from the left anterosuperior iliac spine, including the standard region for iliac crest biopsy [2]. The bone specimens were immediately frozen at –20°C until further used. Subsequently, trans iliac crest bone samples were drilled with a trephine with an internal diameter of 7 mm from the frozen bone at the position normally used for iliac crest bone biopsies, i.e. 2 cm below and 2–3 cm behind the anterosuperior iliac spine (Figure 1) and placed in 70% ethanol [49].

2.2. Conventional μ CT

The bone samples were scanned in ethanol in a μ CT scanner (μ CT35, Scanco Medical AG, Brüttisellen, Switzerland) in high-resolution mode (1,000 projections per 180°) with an isotropic voxel size of 6 μ m, an X-ray tube voltage of 55 kVp, an X-ray tube current of 145 μ A, and an integration time of 800 ms.

The cortical bone situated at either end of the iliac crest bone samples were delineated interactively using the standard software supplied with the μ CT scanner. The 3D data sets were low-pass filtered with a Gaussian filter ($\sigma = 1.3$, support = 2) and segmented with a fixed threshold

filter (480.3 mg HA/cm³), which was the same as previously established when analyzing the trabecular bone from a subset of the bone samples [2].

The cortical thickness (Ct.Th) was calculated as the delineated cortex volume divided by the cross sectional area of the bone samples $Ct.Th = TV/(d^2 \times \pi/4)$, where the diameter d of the cylindrical bone samples were measured using a digital caliper [50]. By digitally swapping the bone and the marrow phase and by applying the standard μ CT scanner software to this inverted image set the cortical porosity (Ct.Po), Haversian canal diameter Ha.Ca.Dm, Haversian canal separation Ha.Ca.Sp, and Haversian canal number Ha.Ca.N were determined [51] using the direct method without parallel plate assumptions [52]. Assuming that the Haversian canals can be represented by cylinders, the number of Haversian canals per area Ha.Ca.Dn can be estimated as: $Ha.Ca.Dn = Ct.Po/(Ha.Ca.Dm^2 \times \pi/4)$ [51]. Quality assurance was performed by weekly (density) and monthly (geometry) scans of the solid-state calibration phantom provided with the scanner.

2.3. SR μ CT

After conventional μ CT the iliac bone samples were investigated using SR μ CT at the beamline for TOMographic Microscopy and Coherent rAdiology experimenTs (TOMCAT) at the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland) [53] with an X-ray beam energy of 35 keV and an isotropic voxel size of 0.65 μ m using local tomography. Before SR μ CT measurements, the samples were removed from the ethanol, left to dry overnight in the hutch, and mounted using hot wax, with the long axis of the bone samples perpendicular to the beam. Sample exchange was facilitated by a robot sample exchange system installed at the beamline [54]. The cortical bone of the iliac crest samples was measured in air with one scan of each cortical plate. The in-plane field-of-view was 1.7 mm in diameter per scan. For each individual volume, 1201 projections were measured with an exposure time of 500 ms. The detector system consisted of a 4.3 megapixel CCD

camera (PCO 2000, PCO AG, Kelheim, Germany) coupled to an Optique Peter microscope system with a $\times 10$ objective lens to magnify the visible light converted with a 20- μm -thick LAG:Ce scintillator. Each scan consisted of 2160 reconstruction containing 2540×2160 image pixels. Since two scans were measured per sample, the total scan time was 20 minutes per bone sample excluding time to mount, align, and set up of the scans. Reconstructions were performed using the GridRec algorithm applying a Hamming filter.

2.4. Image post processing and data analysis

The osteocyte lacunar volumes were calculated using the FIJI software package [55], as we have previously described [56, 57]. The reconstructions were low pass filtered with a 3D Gaussian filter ($\sigma = 1.8$) and segmented using a local threshold value determined per image stack using the optimize threshold function of FIJI [58]. Osteocyte lacunar volumes (Lc.V) were calculated by counting the voxels contained within each lacuna, using a lower limit of $50 \mu\text{m}^3$ in order to exclude noise and canaliculi and upper limit of $2000 \mu\text{m}^3$ in order to exclude larger cavities such as Haversian and Volkmann's canals. Additionally, void space in contact with the sides of the virtual sample volume were excluded to remove cavities containing blood vessels as well as osteocyte lacunae coinciding with drilled bone surfaces. Subsequent analysis of osteocyte lacunar properties was performed using MATLAB (R2010b, MathWorks, Natick, MA). Distributions of Lc.V were made (bin size $3 \mu\text{m}^3$) [26], revealing a substantial tail at lower lacunar volumes in the data. The tail is most likely a combination of noise and canaliculi still remaining after the cut off at $50 \mu\text{m}^3$. In order to extract information on the lacunar volumes without bias from this tail, the Lc.V distributions were fitted using lognormal distributions (supplemental Figure 1). The fitting was performed on Lc.V distributions of the individual cortexes, and on the pooled Lc.V distributions of the two cortexes in each sample. No significant differences were observed in the resulting data

between the two cortexes, and hence the pooled cortex data was used for subsequent analyses. The fitting procedure allowed easy extraction of estimates of mode, median, mean, standard deviation, and skewness of the Lc.V distributions. The results are reported as the mean $\pm$ standard deviation of these properties. Due to the asymmetrical nature of the osteocyte lacunar volume distributions [26, 34], we report all the properties mentioned above. The osteocyte lacunar density (Lc.D) was determined as the ratio of the number of osteocyte lacunae and the measured bone volume. The number of osteocytes was obtained as the integrated distribution describing the lacunar volumes. The bone volumes were calculated by summing the number of voxels representing bone after cleaning the binarized images to remove noise and filling holes not touching the outer surface of the sample.

2.5. 3D visualization

Three-dimensional visualizations were performed on filtered and downsampled data using Amira (version 5.6., FEI Visualization Science Group, Mérignac, France) for the conventional μ CT data and Fiji (Volume viewer) for the SR μ CT data.

2.6. Statistical analysis

Linear regression analysis was carried out using the least square method with in-house developed software. The μ CT derived cortical parameters were determined separately for each cortical shell and the mean of these two measurements were given for each individual. The synchrotron derived osteocyte data from the two cortexes measured in each sample were pooled before fitting the data, as no significant differences were detected when comparing data from the two cortexes fitted individually.

Comparisons between two linear relationships were carried out using in-house developed software as previously described in detail [46, 47]. When comparing individuals younger and older than the mean population age, the comparisons are made using Mann-Whitney tests. In all comparisons, $p < 0.05$ was used as level of significance. Unless otherwise stated all data are given as mean $\pm$ standard deviation.

3. Results

An iliac crest bone sample from a 41-year-old woman is presented in Fig. 1. Fig. 1A shows a 3D volume rendering of the iliac crest bone sample obtained by μ CT, revealing the two cortices separated by trabecular bone. The sub-volumes in the two cortices measured by SR μ CT using local tomography are indicated by red boxes. Fig. 1B shows a 3D rendering of the SR μ CT data collected in the upper cortex of the bone sample shown in Fig. 1A. In Fig. 1C, the osteocyte lacunae and blood vessel canals are 3D rendered from the region shown in Fig. 1B. The osteocyte lacunae (yellow) were analyzed from these SR μ CT data, while the blood vessel canals (red) were analyzed using the μ CT data. Fig. 1D reveals the same region as Fig. 1C, but rotated 90° to highlight the high directionality of the Haversian and Volkmann's canals.

3.1. SR μ CT

The total number of osteocyte lacunae analyzed in the study population was a staggering 5×10^6 . No significant differences were found for the mean Lc.Vs between women ($562 \pm 35 \mu\text{m}^3$) and men ($549 \pm 40 \mu\text{m}^3$). Similarly, no significant difference was observed for the median Lc.Vs between women ($538 \pm 31 \mu\text{m}^3$) and men ($523 \pm 39 \mu\text{m}^3$), or the mode of the Lc.V distributions between women ($495 \pm 30 \mu\text{m}^3$) and men ($478 \pm 48 \mu\text{m}^3$). A non-significant trend of slightly higher values in women was observed for the mean, mode, and median Lc.V, but this did not reach the level of significance. Furthermore, the standard deviation of the Lc.V distributions was not significantly different between women ($194 \pm 43 \mu\text{m}^3$) and men ($199 \pm 67 \mu\text{m}^3$), neither was the skewness of the Lc.V distributions between women ($0.94 \pm 0.17 \mu\text{m}^9$) and men ($0.98 \pm 0.31 \mu\text{m}^9$). No significant difference was detected for Lc.D between women ($15.0 \times 10^3 \pm 2.0 \times 10^3 \text{ mm}^{-3}$) and men ($14.9 \times 10^3 \pm 2.1 \times 10^3 \text{ mm}^{-3}$).

The lacunar properties were evaluated according to age, and Fig. 2A illustrates how the mean Lc.V varies with age. Linear regression analysis revealed that no significant change occurred for Lc.V mean, median, or mode with age for either women or men (Table 1). When pooling the data for both sexes, linear regression still revealed no significant changes with age. A similar conclusion was reached by pooling the data according to sex and grouping the data into younger and older than the mean population age (57.2 years), as done previously by other researchers [23, 28]. No significant differences were found for mean Lc.V (younger $550 \pm 42 \mu\text{m}^3$, older $561 \pm 32 \mu\text{m}^3$), median Lc.V (younger $526 \pm 42 \mu\text{m}^3$, older $535 \pm 28 \mu\text{m}^3$), mode Lc.V (younger $485 \pm 46 \mu\text{m}^3$, older $489 \pm 35 \mu\text{m}^3$), standard deviation of the Lc.V (younger $189 \pm 40 \mu\text{m}^3$, older $204 \pm 67 \mu\text{m}^3$) or the skewness of the Lc.V (younger $0.94 \pm 0.21 \mu\text{m}^9$, older $0.98 \pm 0.28 \mu\text{m}^9$).

The Lc.D did not change significantly with age for either women or men (Fig. 2B). However, when pooling the data for both sexes, linear regression revealed that Lc.D decreased significantly with age ($p = 0.016$). When pooling the data according to sex and dividing the individuals into younger and older than the mean population age, the Lc.D was significantly higher in the younger ($15.7 \times 10^3 \pm 2.5 \times 10^3 \text{ mm}^{-3}$) than the older group ($14.6 \times 10^3 \pm 2.3 \times 10^3 \text{ mm}^{-3}$), $p = 0.022$.

3.2. Conventional μCT

Ct.Po increased significantly with age for both women and men (Fig. 3A, Table 2). The data points for each individual were spread around the fit-lines, but the fit-lines were remarkably similar for women and men. Ct.Th decreased significantly with age (Fig. 3B), Ha.Ca.Dm was independent of age (Fig. 3C), while Ha.Ca.Sp decreased significantly with age for both women and men (Fig. 3D).

Ha.Ca.N i.e. the number of Haversian canals intercepted per length when traveling in a straight line over the cortical shell increased significantly with age for both women and men (Table 2).

Likewise, the number of Haversian canals per bone area $Ha.Ca.Dn$ increased significantly with age for both women and men (Fig. 3E). A few outliers can be observed for $Ha.Ca.Dn$, and in all these cases the cortical shell was very thin. For all these relationships, neither the slope nor the y -axis intercept of the fit-lines differed significantly between women and men indicating similar age-related changes of the microscopic morphological parameters of iliac crest cortical bone for women and men.

Using the regression coefficients in Table 2, a woman lose 6% of her iliac cortical bone volume from 20 to 90 years of age due to increased porosity under the assumption of a constant cortical thickness and 49% due to thinning of the cortex under assumption of a constant cortical porosity. For men, the corresponding numbers are 6% and 39% respectively. Consequently, the loss of iliac crest cortical bone due to cortical thinning is 8 times higher for women and 6 times higher for men than the bone lost due to increased cortical porosity.

We have previously investigated the microstructure of trabecular bone at the iliac crest and found that BV/TV decreased significantly with age for both women and men [2]. If we now extract the BV/TV of the same bone samples as presently studied, and correlate it with the $Ct.Po$, we find that they are only weakly correlated for women ($r = -0.38$, $p = 0.0090$) and uncorrelated for men. Likewise, the cortical thickness $Ct.Th$ was also only weakly correlated to trabecular BV/TV for women ($r = 0.38$, $p = 0.0097$) and men ($r = 0.35$, $p = 0.023$).

3.3. Relationship between osteocyte lacunar properties and cortical microstructure

Correlations have also been made between the properties measured at different length scales (supplemental Table 1). No significant correlations were found between $Lc.D$ and $Ct.Po$, $Ct.Th$, or the Haversian canal geometric properties. There were significant correlations between $Lc.V$ mode and a number of parameters and a few additional significant correlations to the $Lc.V$ median.

Ha.Ca.Dm was significantly correlated to the Lc.V median and mode for men and to both Lc.V mean, median, and mode for the sex pooled values. In all cases correlation coefficients were approximately -0.3 (-0.24 to -0.34) with $p < 0.03$. Similar correlations were seen for Lc.V median and mode with Ct.Po for the pooled data though the correlation only reached significance for the Lc.V mode for women.

4. Discussion

In the present comprehensive study of iliac crest cortical bone, no significant differences in osteocyte lacunar properties were detected between normal women and men. The influence of sex on osteocyte lacunar volume has not previously been investigated. We found that Lc.V did not differ significantly between women and men. Similarly, Lc.D did not differ significantly between women and men, in agreement with the observation of Busse et al. on their smaller data set [29]. Thus, if there are differences in cortical bone quality between women and men, these are not reflected in the size or density of osteocyte lacunae.

In the present study we report an age-pooled mean Lc.V of $562 \pm 35 \mu\text{m}^3$ for women and of $549 \pm 40 \mu\text{m}^3$ for men for human iliac crest bone. Hesse et al. have reported mean Lc.V values in the range of $296\text{--}646 \mu\text{m}^3$ for human jaw bone [21, 34]. Carter et al. have reported a mean Lc.V value of $252 \pm 63 \mu\text{m}^3$ [23], while Dong et al. have reported a mean Lc.V of $410 \pm 150 \mu\text{m}^3$ [59], and Varga et al. have reported a mean Lc.V of $511 \pm 96 \mu\text{m}^3$ [60] for human femoral cortical bone. Hence, Lc.V values show relatively large variations both within and between studies. This could be explained by differences in Lc.V between different skeletal sites, or differences in the voxel size used in the studies. However, further investigations are needed in order to resolve this.

The processes behind the age-related effects on bone are not fully understood, but with the orchestrating role of osteocytes in bone remodeling, they are expected to participate in the aging process. However, the present study did not reveal any significant changes in Lc.V with age in either women or men. In contrast to our results, Lc.V has been reported to be 30% larger in younger (< 50 years) than in older (> 50 years) women in human femoral cortical bone [23]. Milovanic et al. reported fewer hypermineralized osteocyte lacunae in proximal femoral bone in young woman (31 ± 9.5 years) than in old women (84 ± 5.9 years) [61]. The fact that we do not observe an age dependence in Lc.V indicates that there cannot be a significant fraction of osteocyte lacunae with

varying degrees of hypermineralization; i.e. the lacunae are either not hypermineralized or hypermineralized to such a large degree that they are no longer detected as osteocyte lacunae with the present methodology. Our observation of a decline in Lc.D with age is consistent with complete hypermineralization of some osteocyte lacunae with age. The Lc.D was found to decrease with age, however only reaching the level of significance for the sex-pooled data. This is consistent with reports by Mullender et al. who used histomorphometry to establish that Lc.D was smaller for individuals older than 55 years than for younger individuals in trabecular transiliac samples [28] and by Busse et al. who by the same method found that Lc.D decreased with age in both women and men in femoral cortices [29]. On the other hand, in the synchrotron CT study of Carter et al. no age dependence in Lc.D was detected amongst 30 women aged 20–86 years [23].

The analysis of the iliac crest cortical bone at the microscopic level found that Ct.Po increased significantly with age for both women and men. This is consistent with the findings of some previous studies of normal aging iliac crest cortical bone [62, 63], but at variance with the findings of Vedi et al. who found no age-related changes in Ct.Po in either women or men [42] and Melsen et al. who found an increased Ct.Po in women only [43]. In the present study there was no difference in the age-related changes in Ct.Po between women and men, while previous studies either found that Ct.Po was similar for women and men [42, 63] or that it differed between the sexes [43, 62]. The different outcomes of the various studies are not easily explained. Most previous studies of iliac crest cortical bone have been performed using conventional 2D histomorphometry, whereas the present study was based on 3D μ CT. Another possible explanation could be a difference in the homogeneity of the populations studied. The individuals, from whom the bone samples in the present study were obtained, originated from a small homogenous regional area in Denmark suggesting that the present study material is highly homogenous.

In the present study we found that the cortices of the iliac crest bone samples were significantly thinned with age and that this thinning was similar for women and men. This is consistent with the findings of several previous studies [42, 43, 63], but in contrast to other studies who found age-related cortical thinning in either women [62, 64] or men [65] only or that the Ct.Th was independent of age [66].

We found that Ha.Ca.Dm was independent of age in both women and men, but that there were large interindividual variations. Some of this variation is a result of some of the Haversian canals are undergoing active remodeling, while others are not. Our findings are consistent with the findings of Vedi et al. who showed that Ha.Ca.Ar was independent of age [42], whereas Brockstedt et al. found that Ha.Ca.Dm increased significantly with age [62]. The Ha.Ca.Dm values found by these studies using 2D techniques were lower than those found in the present study using 3D methods. Busse et al. investigated iliac crest bone in 15 normal individuals and found a Ha.Ca.Dm of approximately 85 μm using 3D SR μCT , which is closer to the findings of the present study [67]. The remaining difference may be caused by Busse et al. using a higher spatial resolution than herein, and it cannot be precluded that we have missed the thinnest Haversian canals. This is consistent with the higher number of Haversian canals per area reported by Busse et al. than found in the present study.

Ha.Ca.N and Ha.Ca.Dn increased significantly with age while Ha.Ca.Sp decreased significantly with age for both women and men indicating that the relative number of Haversian canals increased with age. This could explain why Ct.Po increased with age while Ha.Ca.Dm was independent of age.

The loss of iliac crest cortical bone due to cortical thinning was 8 times higher for women and 6 times higher for men than the bone loss caused by increased cortical porosity. That the cortical bone loss due to cortical thinning is larger than due to increased cortical porosity is consistent with

previous observations [64]. With aging, the cortical bone at the endosteal envelope is slowly transformed into trabecular bone, so that the inner cortex is slowly being “trabecularized” by Haversian canals that do not close after bone resorption during remodeling [68–72]. However, when the inner cortex is trabecularized, it can no longer be considered as a part of the cortex, and hence the larger Haversian canals that appear with age are not included in the analysis of cortical bone. Hence, it is not surprising that the diameter of the Haversian canals appears to be independent of age, and that the bone loss due to increased porosity is relatively modest compared to the bone loss due to cortical thinning with increasing age. It has even been argued that a transitional zone situated between the cortical and trabecular bone should be added to the analysis [68, 71]. However, it is not trivial to delineate these zones, especially on radiographic images where bone cells, marrow cells, and osteoid are challenging to identify. In addition, the delineation of cortical bone at the iliac crest is further complicated as it is located adjacent to trabecular bone in contrast to e.g. at a mid-diaphysis consisting solely of cortical bone. Therefore, the relative contribution of cortical thinning and cortical porosity to the loss of cortical bone calculated in the present study is of course dependent on the exact demarcation of the cortical bone.

We found that Ct.Po and BV/TV were weakly correlated and that the relationship was significant for women only. This is consistent with the findings of Ostertag et al., where no significant correlation was found between Ct.Po and BV/TV in bone biopsies from men with and without vertebral fractures [39]. Interestingly, the bone mineralization density distributions of cortical and trabecular bone compartments of transiliac crest biopsies have been reported to be correlated in healthy premenopausal women, suggesting a co-regulation of bone remodeling of the compartments within the iliac crest at least for this sex and restricted age-span [9].

Comparison of morphological measures obtained at different length scales showed that only the Lc.V mean, median, and mode were significantly if weakly correlated to the Ha.Ca.Dm. This

indicate that the morphological measures at the two length scales contain different morphological information and are thus both needed for a comprehensive characterization of the bone morphology. We speculate that the negative correlation between Lc.V and Ha.Ca.Dm might be connected to Haversian remodeling processes and this could form the basis for future work in this area. We found that neither Ha.Ca.Dm nor Lc.V depend on age suggesting that the correlation between Lc.V and Ha.Ca.Dm reflects underlying remodeling dynamics rather than an age-connected coupling of the two. The observation of a negative correlation between lacunar volume and canal diameter indicates that much remains to be learned about the formation of Haversian canals in humans. In particular, the coupling of Haversian canal formation and osteoblast incorporation into the bone matrix and osteocyte maturation needs further investigation.

The present study investigated iliac crest bone samples from a very high number of women and men over a very broad age-range. However, the study has nevertheless a few limitations. Due to time limitations at the synchrotron, the study covered cortical bone only. The inter individual variation observed in the present study can be partly explained by the fact that only a sub-volume was investigated, as is always the case with these type of studies. Another limitation is that our study is cross sectional in nature rather than longitudinal, which is a limitation of all autopsy studies. It can not be excluded that if more individuals had been included in the study the slight increase in Lc.V might have been statistical significant. However, as we already have included a very high number of individuals (88) the age-related increase in Lc.V would then have been minute. Finally, we tried to make our population as “normal” as possible through our exclusion criteria, but we did not exclude individuals with osteoporosis. Osteoporosis is characterized by low bone density and often occurs with increasing age. Excluding osteoporotic individuals would therefore have skewed our aging population leading to an underestimation of the age-related changes.

In conclusion, through the investigation of a very large number of individuals of either sex spanning a large age-range, we found that the size of osteocyte lacunae did not depend on either age or sex. Osteocyte lacunar density only decreased significantly with age in the sex pooled population, and did not differ between women and men. Furthermore, cortical porosity and Haversian canal density increased while cortical thickness decreased with age, with cortical thinning dominating the age-related cortical bone loss. None of the cortical microstructural parameters showed any sex dependency. Finally, we found only weak links between osteocyte lacunar properties and cortical microstructural properties in iliac crest bone. The osteocyte lacunar properties and cortical bone microstructural properties determined in the present study are obtained from normal bone at the standard location for clinical bone biopsies. Hence, the age- and sex-related findings based on a large number of individuals will serve as important reference data for future studies in both health and disease.

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HB, and JST. Approving final version of manuscript: FLBG, AB, MVJ, ENE, HB, and JST. HB and JST take responsibility for the integrity of the data analysis. HB and JST are joint senior author.

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Figure legends

Fig. 1. 3D renderings of a 7-mm-diameter iliac crest bone biopsy from a 41-year-old woman. (A) μ CT data showing the two cortices with regions corresponding to those measured by SR μ CT marked in red. (B) The SR μ CT volume collected from the upper region marked in Fig. 1A featuring the outer bone surface in the upper part of the image. (C) Osteocyte lacunae (yellow) and blood vessel canals (red) shown in a direction perpendicular to that presented in Fig. 1A. (D) Osteocyte lacunae (yellow) and blood vessel canals (red) shown in the same direction as that presented in Fig. 1A. The red plate-like shape at the top of Figs. 1 C and D represents the sample surface that was not planar (see Fig. 1B).

Fig. 2. (A) Mean osteocyte lacunar volume, Lc.V, for each individual as a function of age. (B) Osteocyte lacunar density Lc.D as a function of age. Open (filled) circles and dashed (solid) fit-lines represent women (men). Regression and correlation coefficients can be found in Table 1.

Fig. 3. Micro-structural measures as a function of age. (A) Cortical porosity Ct.Po, (B) cortical thickness Ct.Th, (C) mean Haversian canal diameter Ha.Ca.Dm, (D) mean Haversian canal separation Ha.Ca.Sp, (E) Haversian canal number, (F) Haversian canal density Ha.Ca.Dn. Women are shown with open symbols and dashed fit-lines and men are shown with filled symbols and solid fit-lines. Regression and correlation coefficients can be found in Table 2.

Tables

Table 1. Linear regression coefficients (*a*: slope and *b*: y-axis intercept) and Pearson's correlation coefficients (*r*) for the age-related changes in osteocyte lacunar volume Lc.V and density Lc.D for women and men.

		<i>a</i>	<i>b</i>	<i>r</i>	<i>p</i>
Lc.V	Women	0.18	552	0.10	0.490
	Men	0.44	525	0.22	0.164
Lc.V (median)	Women	0.061	534	0.040	0.791
	Men	0.33	504	0.17	0.286
Lc.V (mode)	Women	−0.13	503	−0.085	0.574
	Men	0.19	468	0.078	0.623
Lc.D	Women	−24	16.6×10^3	−0.24	0.103
	Men	−30	16.6×10^3	−0.28	0.070

Table 2 Linear regression coefficients (*a*: slope and *b*: y-axis intercept) and Pearson's correlation coefficients (*r*) for the age-related changes in cortical microstructure for women and men.

		<i>a</i>	<i>b</i>	<i>r</i>	<i>p</i>
Ct.Po	Women	0.084	4.0	0.50	0.00040
	Men	0.085	4.4	0.37	0.017
Ct.Th	Women	-0.0086	1.4	-0.52	0.00023
	Men	-0.0065	1.3	-0.37	0.014
Ha.Ca.Dm	Women	0.16	119	0.10	0.496
	Men	-0.12	136	-0.07	0.651
Ha.Ca.Sp	Women	-1.02	397	-0.53	0.00016
	Men	-0.98	384	-0.48	0.0012
Ha.Ca.N	Women	0.0075	2.5	0.51	0.00027
	Men	0.0081	2.6	0.47	0.0016
Ha.Ca.Dn	Women	0.065	3.9	0.38	0.0096
	Men	0.067	4.1	0.35	0.023

Figure 1

Figure 2

Figure 3