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Sex Differences of Human Trabecular Bone Microstructure in Aging Are Site-Dependent

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ABSTRACT: In this study, we characterize bone microstructure, specifically sex differences, at multiple skeletal sites in 165 subjects >52 yr of age, using μCT technology in vitro. Significant sex differences are observed at the distal radius, femoral neck, and femoral trochanter, but not at the iliac crest, calcaneus, and lumbar vertebral body. Correlations in BV/TV between sites ranged from $r = 0.13$ to 0.56.

Introduction: The goals of this study were (1) to assess potential sex differences of bone microstructure and their difference between skeletal sites and (2) to explore the relationship of trabecular microstructural properties between relevant skeletal sites.
Materials and Methods: Trabecular bone microstructural properties were measured in vitro in 165 subjects 52–99 yr of age using μCT. Defined volumes of interest (cylinders with 6 mm diameter and 6 mm length) were scanned at a resolution of 26 μm (isotropic) in six different anatomical sites: distal radius, femoral neck and trochanter, iliac crest, calcaneus, and second lumbar vertebral body.
Results: At the radius and femoral neck, trabecular bone displayed a more plate-like structure, thicker trabeculae, smaller separation/higher trabecular number, higher connectivity, and a higher degree of anisotropy in men than in women ($p < 0.05$). At the trochanter, men displayed more plate-like structure and thicker trabeculae ($p < 0.05$), but no differences in trabecular separation or other parameters compared with the women. At the calcaneus, iliac crest, and second lumbar vertebra none of the bone parameters displayed significant differences between sexes. The BV/TV at one site explained a range of only 2–32% of the variability at other sites.
Conclusions: These results suggest that trabecular bone microstructural properties are remarkably heterogeneous throughout the skeleton. Significant differences between men and women are observed at some, but not at all, sites. The magnitude of sex differences in trabecular microstructure coincides with that of fracture incidence observed for some of the sites in epidemiological studies.
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Key words: trabecular bone, microstructure, osteoporosis, sex, heterogeneity

INTRODUCTION

Osteoporosis has been defined as a systemic skeletal disease, characterized by a reduction of bone density and quality, leading to a reduction in bone strength and increased susceptibility to fracture.[1] Among the relevant characteristics of bone quality, its architecture (i.e., microstructure) has been suggested, as well as bone turnover, damage accumulation, and mineralization. The status of trabecular bone microstructure in the human skeleton has, however, not been well characterized, and the impact of sex and site on trabecular microstructure remains ill defined. Amling et al.[2] used histomorphometry to study trabecular microarchitecture at the lumbar vertebral bodies, the iliac crest, the femur, and the calcaneus of 12 healthy autopsy cases 28–84 yr of age. They reported a high degree of heterogeneity of bone microstructure with the trabecular bone volume fraction (BV/TV, %) ranging from 8.3% in the lumbar spine to 15.8% in the femoral neck. Parkinson and Fazzalari[3] examined 280 histological sections from eight anatomical sites in 113 human specimens and reported differences between sites, as well as striking variability between subjects at each site. Hildebrand et al.[4] used μCT to derive 3D measures of trabecular microstructure from five

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skeletal sites (femoral head, second and fourth lumbar vertebral bodies, iliac crest, and calcaneus) from 52 donors and also found microstructural properties to vary substantially throughout the human skeleton. These studies did not investigate, however, the correlation of trabecular bone properties between different skeletal sites and sex differences of trabecular microstructure, although it is well known that women and men display differences in the prevalence of osteoporotic fractures.[5–12]

In this study, we therefore investigated sex and site dependence of trabecular bone microarchitecture in a sample of 165 subjects at relevant skeletal sites using μCT technology. We tested the hypothesis that sex differences of BV/TV exist but differ in magnitude between skeletal sites. Additionally, we explored the relationship of trabecular microstructure between relevant skeletal sites and the microstructural characteristics that form the basis of potential differences in BV/TV between women and men in aging.

MATERIALS AND METHODS

Study sample

We studied a total of 168 embalmed human cadavers from a series of three consecutive courses of macroscopic anatomy. The donors had agreed to dedicate their body to the Institute of Anatomy at the LMU München several years before death for educational and research purposes in line with local legislative requirements. Bone biopsies were taken from the right iliac crest (at the site of clinical transiliac biopsies) and prepared for routine histomorphometric assessment (embedding in methylmethacrylate, preparation of 5-μm sections, staining with Goldner, Toluidin blue, and von Kossa). Three specimens with signs of malignancy were discarded from the study, so that a total of 165 specimens were left for analysis (age range, 52–99 yr; 79 women 81.2 ± 9.0 yr of age and 86 men 79.1 ± 9.9 yr of age). To be able to compare the impact of sex on trabecular microstructure independent of age, 75 women (age 80.8 ± 9.0 yr; range, 53–98 yr) and 75 men (age, 80.8 ± 9.0 yr; range, 52–99 yr) were selected posthoc on a paired 1:1 basis so that the age distribution of both samples was closely matched. The age difference was ≤1 yr in 66 paired samples and ≥1 yr in only 9 paired samples, with the minimal and maximal paired difference being –1.6 and +1.8 years, respectively (men versus women).

Techniques used for sample collection

In all 165 subjects, the following additional bones were collected: left pelvis, proximal femur, calcaneus, distal radius, and second lumbar vertebra (L_2). If one of these bones was not available (e.g., previous fracture or total hip replacement), the contralateral side was used. The vertebrae, femora, and radii were radiographed in two planes to exclude previous fracture using a Polyphos 30 M X-ray system (Siemens, Erlangen, Germany). In the spine, four films were obtained (two in anterior-posterior and two in lateral projection): one set focusing on the thoracic and one set on the lumbar region. Images were analyzed with regard to the presence of spinal fractures by a musculoskeletal radiologist (TML), using a semiquantitative score according to previously published guidelines[13] with vertebrae displaying deformities > grade 1 being excluded from the μCT analysis. Cylindrical specimens were retrieved using diamond trephines (Salzmann, Munich, Germany) as described previously.[14] A drill bit with a 12-mm inner diameter was used at the iliac crest at the site of clinical transiliac biopsy,[2] the length of the sample being determined by its natural width. An 8-mm trephine was used at all other sites. In L_2, a full-length cylinder was obtained in the superior-inferior direction at 50% of the medio-lateral length of the vertebra (the middle) and at the transition of the anterior third (33%) to the posterior two thirds (66%) of the anterior-posterior length, to avoid the posterior venous plexus. A 14-mm-long specimen was obtained from the center (superior-inferior direction) of the full-length cylinder. The orientation of the trabeculae in the femoral neck was determined from an anterior-posterior contact radiograph, and a 14-mm planoparallel section was obtained from the femoral neck using an high precision band saw (EXAKT Trennschleifsystem; Otto Herrmann, Norderstedt, Germany). The section was obtained in the middle of the femoral neck, perpendicular to the primary trabecular orientation of each individual femur.[14] This section was radiographed again to identify the main trabecular bundle within the section. Eventually a cylindrical specimen was retrieved at this site. In the trochanter, a 14-mm section was obtained in a direction perpendicular to the direction of a fall on the greater trochanter (10° adduction, 15° internal rotation).[15–17] This section was radiographed and a cylindrical specimen (8 mm × 14 mm) retrieved from the dense central region of the section, perpendicular to the slice and parallel with the impact direction during a fall on the side.[15–17] In the calcaneus, a cylinder was obtained in the medio-lateral direction at 50% of the height of the calcaneus (middle) and at the transition of the anterior two thirds (66%) and posterior thirds (33%) of the calcaneus.[18] A 14-mm-long specimen was obtained from the center (medio-lateral direction) of the full-length cylinder. In the distal radius, a 14-mm section was retrieved at the distal metaphysis, perpendicular to the long axis of the shaft. The distal end of the section was located 2 mm proximal from the wrist joint cavity,[19] and a cylindrical specimen was finally obtained in the center of the section. The samples were stored in a solution of 5% buffered formalin until μCT scanning.

For technical reasons, it was not possible to obtain a sample from each site, because no specimens were harvested from bones with signs of previous fracture, because in some cases bones were not available (e.g., bilateral hip endoprosthesis) and because some samples disintegrated during the retrieval. Some calcaneal specimens were used for another study and were therefore not available. The total number of specimens in females (f) and males (m) that were finally scanned at each site were as follows: $n = 163$ at the left pelvis (78f/85m), 134 at L2 (61f/73m), 145 at the femoral neck (65f/80m), 151 at the femoral trochanter (71f/80m), 128 at the calcaneus (64f/64m), and 162 at the distal radius (78f/84m). Those included in the age-matched analysis amounted to $n = 148$ at the left pelvis (74f/74m), 122 at L2 (58f/64m), 133 at the femoral neck (63f/70m), 138 at the

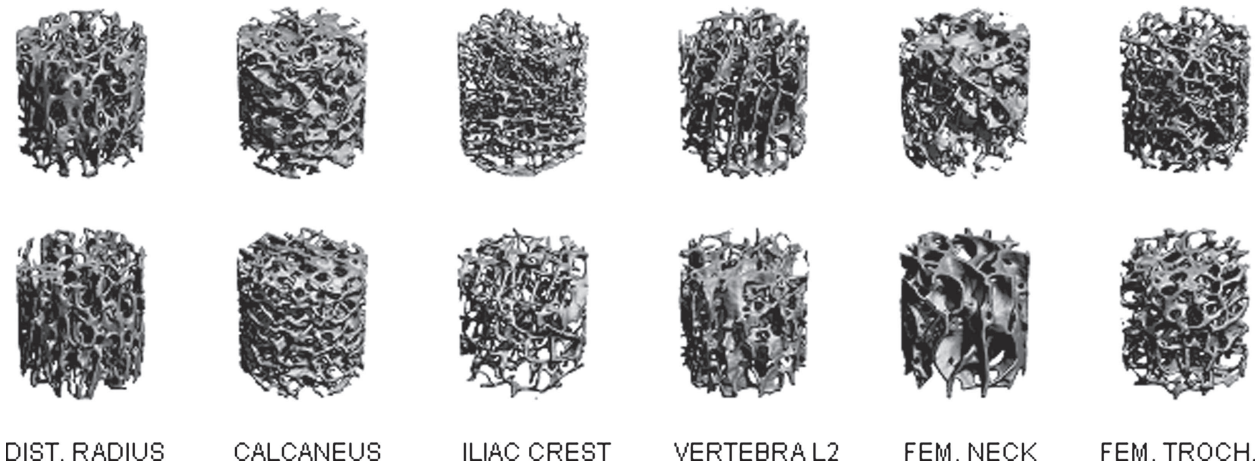

FIG. 1. Reconstructions of bone microstructure from μCT scans at the six skeletal sites studied, with examples of average properties shown for women (top) and men (bottom).

femoral trochanter (68f/70m), 117 at the calcaneus (61f/56m), and 147 at the distal radius (74f/73m).

μCT scanning

The scans were acquired for the central 6 mm of the specimen (Fig. 1) using a μCT 20 scanner (Scanco Medical, Bassersdorf, Switzerland) as described previously.[14] In brief, the resolution was set to 26 μm (isotropic), similar to a previous study on human trabecular bone,[4] with "medium" scan mode and at an integration time of 100 ms. The total scan time per sample was 4.1 h. Within a defined volume of interest (VOI: diameter, 6 mm; length, 6 mm; Fig. 1) we determined the following 3D structural parameters, using the following settings (Sigma 0.8; Support 1.0; Threshold 22% of maximal gray value) and the software provided by the manufacturer: (1) bone volume fraction (BV/TV, %); (2) trabecular number (Tb.N; 1/mm); (3) trabecular thickness (Tb.Th; μm); (4) trabecular separation (Tb.Sp; μm); (5) structure model index (SMI),[20] a measure of plate- or rod-like trabecular architecture; (6) connectivity density (Conn.D; $1/mm^3$), and (7) degree of anisotropy (DA). Note that an ideal plate structure displays an SMI of 0 and a rod structure an SMI of 3, independent of the physical dimensions. All above parameters were computed in 3D without model assumptions required for 2D-based analysis.[4] When repeating analyses again 8 wk later, we showed that the μCT measurements are highly reproducible (range of the root square mean [RMS] CV%, 0.64–1.29% for BV/TV at different sites) with the device settings mentioned above. Displacements of the VOI of up to 4 mm generally lead to nonsignificant systematic differences in mean values of <10%.

Statistical analysis

Sex differences at the various skeletal sites were first evaluated for statistical significance using an unpaired, two-sided *t*-test, using the age-matched sample ($n = 150$) with a level of significance of $p < 0.05$. Repeated-measures ANOVA with sex as a fixed factor was used to evaluate interaction effects between sex and skeletal sites. Pearson correlation coefficients between the sites were evaluated by simple linear regression analysis in the total sample ($n = 165$) and for women ($n = 79$) and men ($n = 86$) separately.

RESULTS

The BV/TV was lowest in the iliac crest (6.6 ± 3.0% in men and 7.1 ± 3.6% in women), and for the men was highest in the femoral neck (17.6 ± 9.3%), whereas in women, it was highest at the calcaneus (14.0 ± 4.9%). Women displayed significantly lower values of BV/TV than men at the distal radius (–30%; $p < 0.001$), at the femoral neck (–35%; $p < 0.001$), and at the femoral trochanter (–19%; $p < 0.001$), but not at the calcaneus (–8%, $p = 0.25$), the iliac crest (+7%; $p = 0.36$), and at L_2 (–9%, $p = 0.19$; Fig. 2A). The interaction between sex and site was statistically significant ($p = 0.00006$).

The iliac crest displayed the most rod-like trabecular bone structure (SMI = 2.33 ± 0.54 in men and 2.35 ± 0.54 in women). The most platelike structure was observed for men at the femoral neck (SMI = 1.27 ± 0.89) and for the women at the calcaneus (SMI = 1.66 ± 0.49). Sex differences were significant at the distal radius, femoral neck, and trochanter, but not at the other sites (Fig. 2B), with the interaction effect (sex and site) being statistically significant ($p = 0.0003$).

The DA was highest in the distal radius (2.0 ± 0.28 in men and 1.87 ± 0.30 in women) and femoral neck (2.0 ± 0.39 in men and 1.85 ± 0.35 in women) and lowest in the iliac crest (1.39 ± 0.16 in men and 1.37 ± 0.13 in women). Sex differences were only significant at the distal radius and the femoral neck but not at the other sites (Fig. 2C); the interaction effect did not reach statistical significance for DA ($p = 0.21$).

The trabeculae were thickest in the femoral neck (182 ± 46 μm in men and 166 ± 32 μm in women) and thinnest in the iliac crest (126 ± 19 μm in men and 129 ± 21 μm in women), with sex differences being significant at the distal

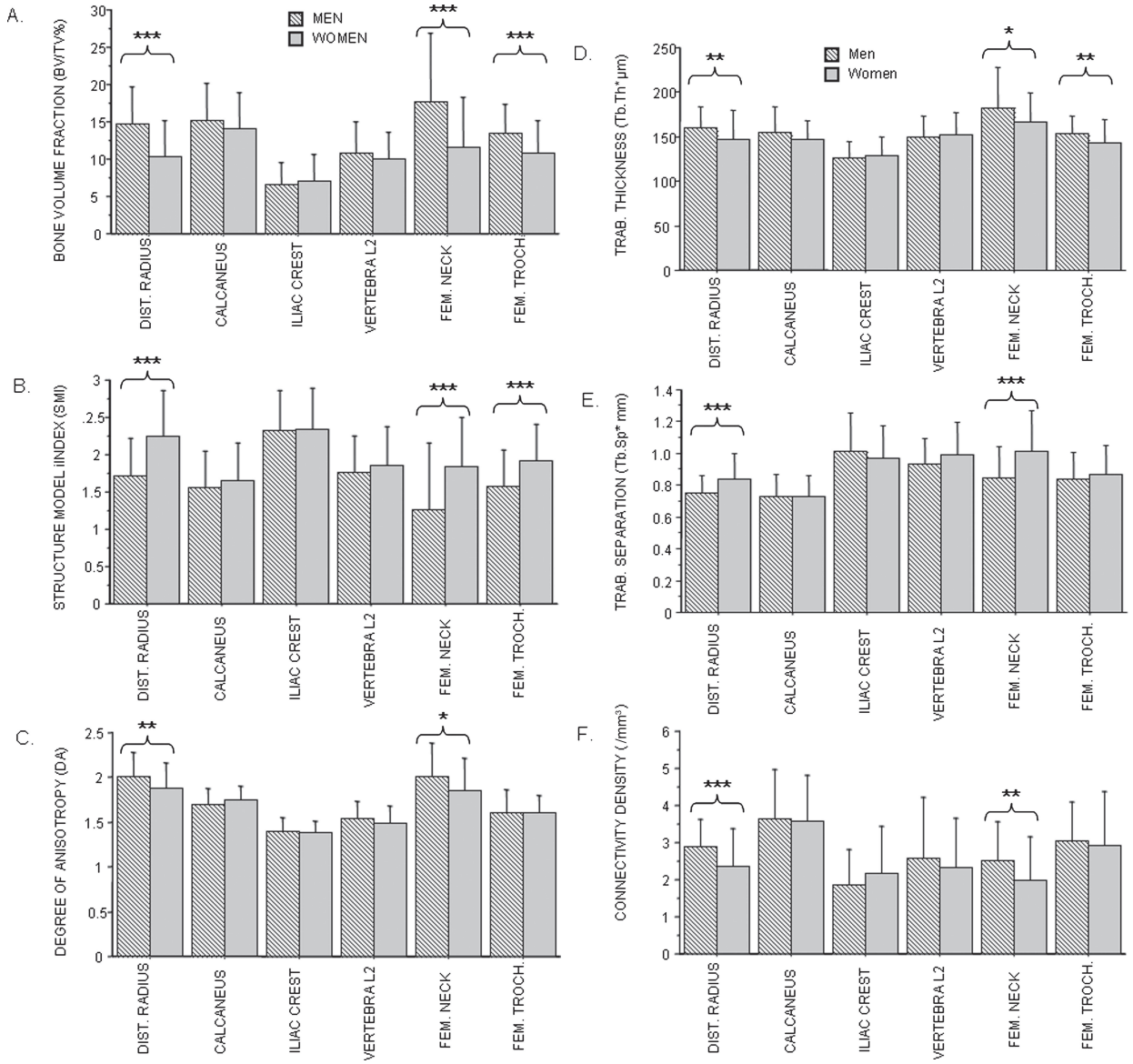

FIG. 2. Bar graphs showing the mean value and SD of bone microstructural properties at six skeletal sites for 75 men and 75 age-matched women (80.8 ± 9.0 yr). (A) Bone volume fraction (BV/TV). (B) Structure model index (SMI). (C) Degree of anisotropy (DA). (D) trabecular thickness (Tb.Th). (E) trabecular separation (Tb.Sp). (F) Connectivity density. Results for trabecular number are not shown because they scale inversely with trabecular separation. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (unpaired t-test between men and women).

radius, femoral neck, and femoral trochanter, but not at the other sites (Fig. 2D). Trabeculae displayed the lowest degree of separation (729 ± 141 µm in men and 726 ± 132 µm in women; Fig. 2E) and highest number (1.30 ± 0.20/mm in men and 1.31 ± 0.19/mm in women) at the calcaneus. In men the highest degree of separation and lowest number of the trabeculae was observed at the iliac crest (1015 ± 239 mm and 0.99 ± 0.17/mm), whereas in women, this applied to the femoral neck (1012 ± 255 mm and 0.99 ± 0.22/mm). Sex differences were highly significant at the distal radius and femoral neck, but not at other sites (Fig. 2E). Interaction effects (sex and site) were $p = 0.041$ for trabecular thickness, $p = 0.0001$ for trabecular separation, and $p = 0.002$ for trabecular number.

The connectivity density was highest at the calcaneus (3.61 ± 1.34 /mm^3 in men and 3.56 ± 1.25 /mm^3 in women; Fig. 2F). In men, it was lowest at the iliac crest (1.85 ± 0.95/mm^3) and in women at the femoral neck (1.98 ± 1.18/mm^3). Again, sex differences were only significant at the distal radius and femoral neck, but not at other skeletal sites, and the interaction effect was statistically significant at $p = 0.03$.

The correlations between sites (Table 1) were only moderate, with the highest correlation in BV/TV being observed between the distal radius and the calcaneus ($r = 0.56$; Fig. 3A) and the lowest between L_2 and the femoral neck ($r = 0.13$; Fig. 3B). The correlations were not strikingly different when analyzing men and women separately,

Table 1. Pearson Correlation Coefficients of Trabecular Bone Microstructural Parameters Between Different Skeletal Sites in 165 Subjects (79 Women and 86 Men) 52–99 yr of Age

	BV/TV	*CD*	*SMI*	*Tb.N*	*Tb.Th*	*Tb.Sp*	*DA*
Rad–calc	0.56*	0.48*	0.45*	0.55*	0.42*	0.51*	0.21*
Rad–iliac	0.25*	0.26*	0.20*	0.42*	0.23*	0.38*	–0.05
Rad–L_2	0.39*	0.26*	0.31*	0.36*	0.31*	0.43*	0.20*
Rad–neck	0.25*	0.33*	0.20*	0.32*	0.26*	0.35*	0.05
Rad–tro	0.34*	0.28*	0.36*	0.44*	0.31*	0.40*	0.03
Calc–iliac	0.38*	0.35*	0.26*	0.38*	0.16	0.38*	–0.10
Calc–L_2	0.28*	0.21*	0.28*	0.24*	0.33*	0.28*	0.20
Calc–neck	0.23*	0.32*	0.16	0.33*	0.20*	0.33*	0.05
Calc–tro	0.39*	0.28*	0.30*	0.33*	0.39*	0.32*	0.01
Iliac–L_2	0.27*	0.18*	0.34*	0.41*	0.28*	0.48*	–0.08
Iliac–neck	0.16*	0.37*	0.08	0.29*	0.15*	0.33*	0.08
Iliac–tro	0.18	0.13	0.24*	0.17*	0.17	0.17*	–0.04
L2–neck	0.13	0.09	0.17	0.22*	0.10	0.30*	0.17
L2–tro	0.20*	0.17*	0.23*	0.20*	0.23	0.22*	0.07
Neck–tro	0.31*	0.28*	0.32*	0.28*	0.24*	0.30*	0.08

* Significant at $p < 0.05$.

BV/TV, bone volume fraction; CD, connectivity density; SMI, structure model index; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; DA, degree of anisotropy; Rad, distal radius; Calc, calcaneus; Iliac, iliac crest; L_2, second lumbar vertebral body; Neck, femoral neck; Tro, femoral trochanter.

with coefficients for the BV/TV ranging from $r = 0.05$ (L_2 versus femoral neck) to 0.55 (radius versus calcaneus) in men and from $r = 0.15$ (L_2 versus femoral neck) to 0.57 (radius versus calcaneus) in women. The correlation between the femoral neck and trochanter (BV/TV) was only 0.31 in the total sample (Table 1) and was 0.18 in men and 0.36 in women, respectively.

DISCUSSION

In this study, we tested the hypothesis that sex differences of bone microstructure differ in magnitude between skeletal sites and explored the relationship of trabecular microstructure between relevant skeletal sites and the microstructural characteristics that form the basis of differences in BV/TV between women and men in aging. We found that, at the distal radius and femoral neck, all microstructural properties differ significantly between men and women, the bone displaying a more platelike structure, thicker trabeculae, more trabeculae/smaller separation, a higher connectivity, and a higher degree of anisotropy in men. At the femoral trochanter, men also displayed more platelike structure and thicker trabeculae than women, but there existed no significant differences in trabecular separation/number, the connectivity density, and DA. At the calcaneus, the iliac crest and L_2 bone microstructural properties were remarkably similar between sexes and displayed no significant differences between men and women. At the distal radius and femoral neck, differences in both trabecular thickness and trabecular separation/number explain the sex differences in BV/TV, whereas at the femoral trochanter, only the differences in trabecular thickness (but not those in trabecular separation/number) were significant. Significant interaction effects were found for all microstructural parameters except for DA, showing that the observed sex differences depend on the anatomical sites studied. Microstructural properties displayed not only differences, but also a substantial degree of heterogeneity (lack of correlation) throughout the skeleton, with the BV/TV of one site explaining a range of only 10–32% of the variability at other skeletal sites. This heterogeneity was found both in men and women.

The strengths of this study are that a high number of specimens was investigated from multiple skeletal sites in the same subjects, that the subjects can be assumed to be a representative cross-section of the population at this age in this region, that the men and women were precisely age matched, and that all specimens were studied with conventional histology, to rule out other bone diseases than osteopenia or osteoporosis. Limitations of the study include the limited information on the medical history of the subjects and the limited size of the bone biopsies that were harvested and measured (6 × 6 mm). Great care was taken to harvest the specimens at exactly the same anatomical location in all subjects,[14] but the size of the biopsies could not be adapted to the individual size of the bone; therefore, in subjects with large bones, the biopsy covered a smaller region than in those with small bones. Scanning larger samples would have implied excessive scan times with the device in use. On the other hand, the μCT scanner used in this study has the advantage that measurements have been previously validated versus histomorphometry[21,22] and that it was also shown to provide an excellent scan/rescan precision.[14]

The results do not only reveal differences, but also a high degree of heterogeneity (lack of correlation), in trabecular bone microstructural properties between skeletal sites. Because osteoporosis and age-related bone loss are generally viewed as a systemic process,[23] a higher correlation between the sites may have been expected, but other studies have also revealed a substantial degree of skeletal heterogeneity in bone microstructure,[2,4,24] bone mass,[25]

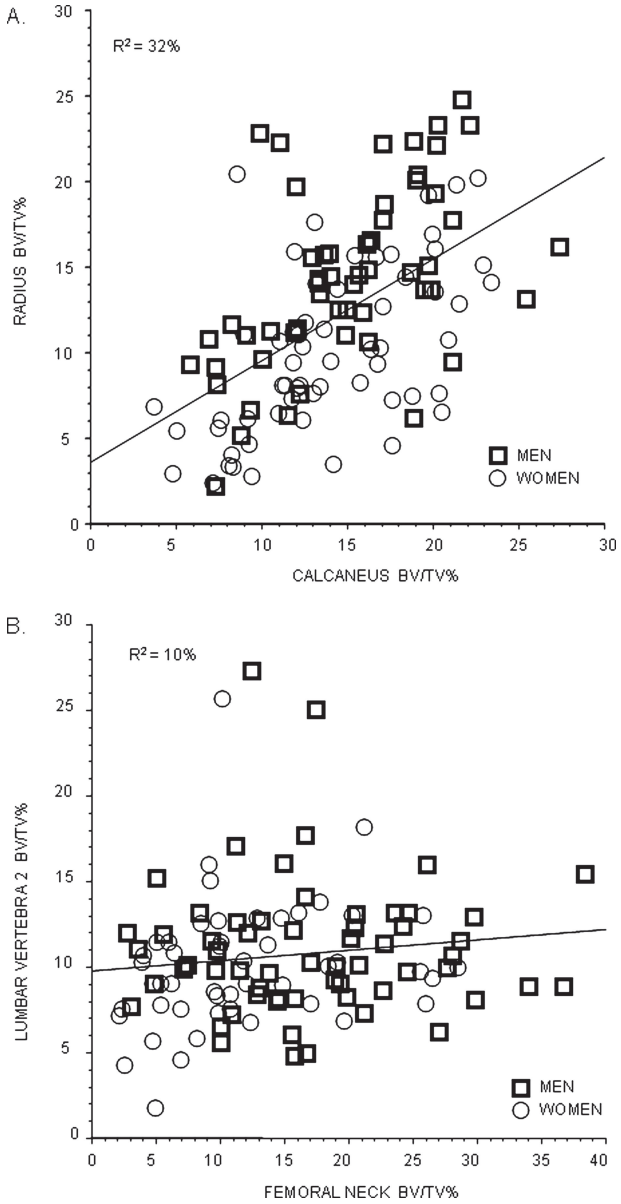

FIG. 3. Bivariate scattergram showing the correlation of bone volume fraction (BV/TV): (A) between the distal radius and calcaneus and (B) between the femoral neck and the second lumbar vertebral body.

BMD,[26] and the mechanical competence of bone.[24,27] The correlations between microstructural parameters amongst different sites in our study are lower than those reported for BMD measurements made with DXA by previous authors.[25,27] This is, however, understandable, because BMD measured with DXA is measured in grams per centimeter squared and scales with bone size. Because larger people display larger bones at all skeletal sites, parameters that are size-dependent (e.g., BMD) naturally display stronger correlations between site than those that are not (e.g., those measured with μCT).

When analyzing bone microstructure at different sites using μCT, Hildebrand et al.[4] observed the femoral head to display the highest BV/TV, thickest trabeculae, and most platelike structure among the sites studied; the lumber spine (L_2) yielded the lowest BV/TV, the thinnest trabeculae, and the most rodlike structure, and the iliac crest and calcaneus showed intermediate BV/TV and structure. The BV/TV values found in our study are similar to those reported by these authors,[4] although the individuals studied by us were on average ~13 yr older. In contrast to their study, however, we found the iliac crest to display the lowest BV/TV and highest SMI, whereas the spine displayed intermediate BV/TV and structure.

Rupprecht et al.[28] analyzed sex differences in trabecular bone microstructure in age matched calcanei of women and men ($n = 15$, respectively) using histomorphometry. They observed men to display somewhat higher values of BV/TV, in particular in the middle age group (40–60 yr) at a superior region of interest in the calcaneus. The region of interest examined in our study, however, coincided with their posterior region of interest, where the authors also found no obvious sex differences. Using high-resolution pQCT, Khosla et al.[29] showed that, at the distal radius, sex differences in bone microstructure exist already in younger individuals (20–29 yr), with young men displaying greater BV/TV and trabecular thickness than women of the same age, but similar values for trabecular number and trabecular separation.

Our finding that no sex differences are apparent at the spine is in contrast to DXA measurements at this site, because these have generally reported higher BMD values in men.[27,30–32] As mentioned previously, however, BMD is measured in grams per centimeter squared with DXA and scales with bone size, with men displaying significantly larger vertebral bodies than women.[33,34] When measuring the volumetric density of vertebral bodies (g/cm^3) with QCT, several studies have reported no differences in density between men and women.[34,35] Sigurdsson et al.,[36] however, recently reported significantly lower trabecular density in women 67–69 yr of age at the lumbar spine and at the proximal femur with QCT, but the sex differences in the femur (–35%) exceeded those in the spine (–17%). A study by Bouxsein et al.,[37] also using QCT, reported significantly higher trabecular density in young women versus young men (age, 21–29 yr), but lower values in women 70–97 yr of age.

Site differences in bone microstructure likely reflect differences in the type and magnitude of loading of trabecular bone and should not be misinterpreted in the sense that sites with lower TB/TV are at higher risk of sustaining fractures. However, the magnitude of sex differences in trabecular microstructure may be related to sex differences in fracture incidence between different sites. Studies in the community population of Rochester, MN, reported that the female:male ratio of fractures in subjects ≥35 yr of age was 4.8:1 at the distal forearm, lower in vertebrae (2.4:1), and between 2.1:1 for intertrochanteric and 2.4:1 for cervical femoral fractures, respectively.[38] When looking only at subjects ≥85 yr of age, however, the female:male ratio shifted to 1:0.92 in the spine (because vertebral fractures increased dramatically near the end of life in men), and the ratio was 1.58:1 (female versus male) in the proximal femur.

In the distal radius, fracture in women outnumbered those in men by far also at high age. A more recent epidemiological study on osteoporotic fractures in the United Kingdom[39] found a ratio of ~4:1 (female versus male) at the distal radius around age 80, a ratio of ~2:1 in the proximal femur, and a similar incidence of fractures of the spine in men and women. Our current findings of sex-specific differences in bone volume fraction and structure in the distal radius and proximal femur, and the absence of these differences in lumbar vertebrae in the same sample of subjects 80 yr of age on average, support the view that differences in bone morphology and mechanical competence may be responsible for the sex-specific fracture rates at different skeletal sites, with a higher female:male ratio of fractures in the distal radius and femur than in vertebrae.

In conclusion, this study showed that, at the distal radius and femoral neck, trabecular bone displays a more platelike structure, thicker trabeculae, smaller separation, higher connectivity, and a higher degree of anisotropy in men than in women, whereas at the calcaneus, iliac crest, and L_2, none of the parameters differed significantly between sexes. The BV/TV at one site explained a range of only 2–32% of the variability at other sites, showing that trabecular bone microstructure is remarkably heterogeneous throughout the skeleton of subjects of advanced age. The magnitude of sex differences in trabecular microstructure coincides with that of fracture incidence observed for some of the sites in epidemiological studies.

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