Addendum: pQCT as an investigation tool

Introduction

Density

There is an increasing interest for non-invasive investigations on cancellous bone. This holds true for the clinical and for laboratory investigations. Nowadays, the bone mineral density (BMD) at almost all skeletal sites (hand, wrist, hip, spine……) is readily accessible in vivo (Genant et al. 1996).

Basically these tools measure a physical property of bone: its apparent density. If one takes 1 cubic centimeter of bone (be it cortical or cancellous bone), removes all the non-bone material (fat, marrow, vessels), he can measure the amount of bone present in this sample. The amount of bone can be measured in different ways. The sample can be weighed when wet (wet apparent density, g/cm³), but this technique depends on the method used to remove the marrow without drying the bone tissue (Carter and Hayes 1977). Alternatively, the sample can be dried (Hansson et al. 1987) or freeze dried to remove all the water and then weight. This gives the dried apparent density (that we used in this study, g/cm³). Finally, the sample can be ashed in an oven (> 400° C) to remove water and the organic phase. This gives the ash apparent density which is the equivalent of the BMD. The term 'apparent' means that the volume to which the density measurement refers is the external volume of the sample, not the volume of bone tissue. For cancellous bone there is an excellent correlation between wet apparent density, dry apparent density, BMD and bone volume fraction (BV/TV) (Banse and Devogelaer 2001). The reason for this is quite clear. First the water content of bone tissue is quite constant (16 to 19%). Second the mineral content of bone tissue is also very stable, as the mineral accounts for 62 to 65% of the dry weight of bone tissue (Hansson et al. 1987; Galante et al. 1970; Mueller et al. 1966; Arnold 1960).

Regarding the cancellous bone, with aging or osteoporosis, the trabeculae do not demineralize. There is just less and less bone tissue: trabeculae disappear or get thinner. So, most of the above mentioned parameters (BV/TV, wet, dry and ash apparent density or BMD) are somewhat interchangeable for the cancellous bone—i.e., the higher the porosity, the lower the density.

All non-invasive density measurement tools (SXA, DXA, QCT, pQCT…) are based on the principle that when the X-ray (or photon) beam passes through the bone, its intensity is attenuated by the mineral. As mineral has a high atomic number compared to soft tissue or air, high density is associated with a high attenuation of the beam. Nevertheless, the density data collected from these tools *should always be validated and calibrated*, referring to an accurate external measurement (be it wet, dry or ash apparent density, or BV/TV).

Microarchitecture

Beside a correct measurement of density, some non-invasive tools (those that make tomographic images, MRI, HRCT, pQCT, μCT) give some access to the structural organization of the trabeculae (Genant et al. 1996). However, the basic concepts for the description of bone microarchitecture (like thickness, connectivity) have been originally designed for (and on) thin histological sections. So, like the density measured with a caliper rule and a balance is the gold standard for density, histology is the gold standard for the morphometry. 'Non-invasive' imagery machines should—ideally—be compared to a histological standard.

The pQCT

In part I, we measured the density of the 136 bone cylinders with a pQCT (for peripheral Quantitative Computed Tomography). The pQCT is an X-ray scanner based on the translation-rotation principle (Figure 26). It has a relatively small gantry (compared to the usual CT scan) allowing the assessments of small objects (< 8 cm in diameter). The major difference with clinical CT scans is that it has a smaller gantry (8 cm vs 80 cm) and rela-

Figure 26. pQCT scanner used for this study.

tively higher resolution (70µ vs 200µ). The major difference with µCT (micro CT) is that is has a larger gantry (for µCT typically ~1cm) a lower resolution (70µ vs 20µ). Most of all, the pQCT produces images (slice or tomography) where, for each pixel, the mineral density is quantitatively measured. The µCT does not have that property since each pixel is only assigned as being a bone or a non-bone pixel.

The pQCT has been widely used in the clinical field for investigation of the shape and density of the distal radius (Boonen et al. 1997). Some models have been adapted for the in-vivo investigation of small animals (rats, mice (Turner et al. 2001)…). or morphology (Pistoia et al. 2001; Jiang et al. 1998; Van Rietbergen et al. 1998). On the other hand, such tools have been used for the in vitro investigation of human cancellous bone density (Bailey et al. 1999; Ebbesen et al. 1997).

Purpose

In this study we had some access to the density and morphology using standard techniques (weigh scale or histology) and corresponding pQCT data.

Consequently, our working hypotheses were

1. The pQCT estimates of the density are highly correlated to the reference values
2. The pQCT allows a correct estimation of cancellous bone morphometry. More specifically the pQCT can enhance differences between the subject.

To our knowledge, there is no validation study on human material comparing histological sections with the pQCT morphological assessment. This has been done for µCT (Muller et al. 1996) but not for pQCT.

Material and methods

pQCT image acquisition

A pQCT Research SA+® (Stratec, Pfrorzheim, Germany) was used to scan the 136 cylinders. They were gently pushed in a appropriately sized plastic vial (internal diameter: 8.5 mm) just after extraction by the coring tool. There was no added liquid in the vial. Although most of the marrow was kept during coring (as needed for mechanical testing) some of it spontaneously flew out. Consequently some air bubbles were trapped between the wall of the plastic tube and some trabeculae or within the sample. Using the positioning software of the machine, the volume of the sample was divided from the bottom to the top in four equal parts. In the middle of each of these parts a pQCT image acquisition was done leaving four *slices* (named slice 1, 2, 3 and 4: see figure 27). The slice thickness was 300µ and the pixel size was 70µ by 70µ. To accommodate this resolution the translation of the x-ray source was very slow (1mm/sec) and each image was computed from 360 projections. In these conditions, the overall scan time of a cylinder was about 15 minutes. A daily calibration was performed to check the stability of the signal.

The images generated by the pQCT (272 images) were exported and converted to TIFF files (8 bit, 256 gray levels, cortical bone about 200) using NIH Scion Image® (Scion corp., Frederick, MD). Pixel size of the images was 70µm.

Density measurement with the pQCT

After the acquisition, the 544 images were analyzed with the built-in XCT 5.4 software of the pQCT. A large region of interest (ROI), wider than the cylinder, was chosen and automatically copied to all the images. Further processing was performed within the ROI, where each pixel had a given bone mineral density (BMD, given by the

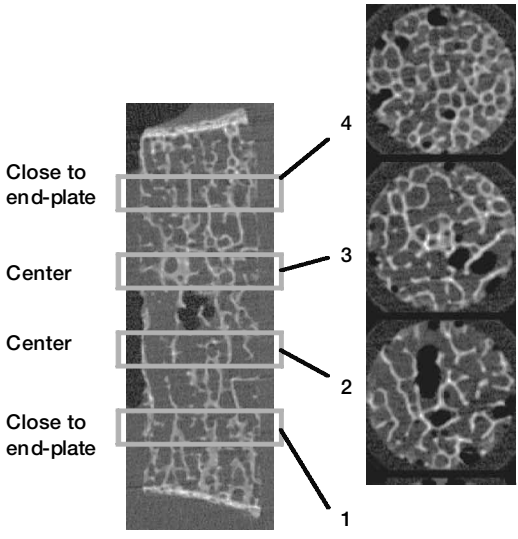

Figure 27. Typical pQCT appearance of the sample. As for the thin histological sections (see fig 11), four transverse slices were obtained from the bottom to the top of each cylinder.

machine as mgHA/cm³) inversely proportional to the attenuation of the X-ray beam within the corresponding voxel. An image segmentation (understand a selection of a sub-population of these pixels) was done using a constant threshold value of 200 mgHA/cm³. This thresholding procedure eliminates all pixels corresponding to air (value < 0 mgHA/cm³) or saline (value: 60 mgHA/cm³ on the pQCT). A previous study had shown that this was an optimal value (Banse and Devogelaer 2000) Consequently, no pixel outside the external perimeter of the cylinder was selected, and the region of interest was automatically and exactly restricted to the sample.

The bone mineral density as measured by the pQCT (pQCT BMD, mgHA/cm³) was calculated as the mean BMD of all the selected pixels multiplied by the proportion of surface that was selected by the thresholding procedure. Practically this proportion was the surface of selected pixels (mm²) divided by the cross-sectional area of the sample (52.8 mm²).

Apparent density

We measured the physical or true apparent density on the 68 samples from one side that have been used for the biochemical analysis (see part III). The end-plates were removed to only consider the volume that was mechanically tested and assessed by pQCT. Bone marrow was removed with a water jet and the samples were defatted. All samples were then freeze-dried, and weighed to give the dry weight (W_D, g). Their height (without end-plates) was measured twice with a caliper (Mitotoyo, UK). The volume of the cylinders was calculated as the product of their height by their cross-sectional area (constant 52.8 mm²). The apparent density (ρ_{app}, g/cm³) was obtained using the formula: W_D / volume.

Architectural parameters

For each of the 272 Von Kossa thin sections described in part II, we analyzed the corresponding pQCT image. This was done on the same computer with the same image analysis software, running the same macro. Only the segmentation of the original image was different: for the pQCT, we used a 'whiter than 85' constant threshold, while for the histological images it was a 'darker than 100' threshold.

Data processing and statistical analysis

Density values (ρ_{app}) were compared to the mean of the four pQCT BMD values of a given cylinder using linear regression.

To estimate the relative accuracy or limitations of the pQCT images to provide a correct morphometrical assessment of a given cylinder, results coming from the four slices of each of them were grouped in a single mean value (68 x 2 average values for the 68 cylinders). Linear regression analysis was then used to estimate the correlation between pQCT and histology.

We also compared the capacity of the two types of investigations to point out differences among subjects using η^2 values. This procedure is described in part II.

Results

Measuring density

The pQCT gave an excellent estimation of the apparent density of the cylinders (Figure 28).

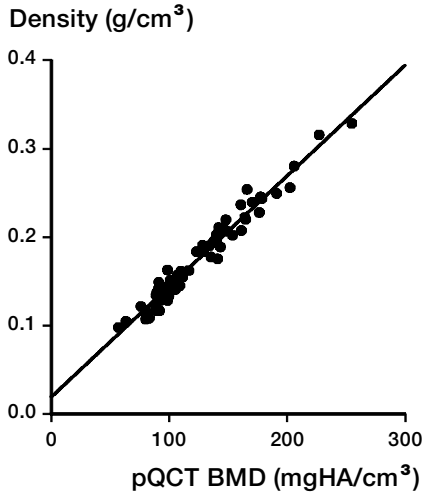

Figure 28. The pQCT gave a very good estimate of density.

Measuring morphometry

On gross examination the pQCT images demonstrated similar morphological characteristics as seen on corresponding histological thin sections (Figure 29).

The BV/TV values obtained with the pQCT were two times higher than on the thin sections (16% vs 8%). On analyzing the pQCT images, the trabeculae appeared two times thicker than their histological control (Tb.Th 161 µm vs 83 µm). The Tb.N was not over-estimated by the pQCT and Tb.Sp slightly underestimated (the difference, 100 µm corresponds to the increase in thickness of the trabeculae). For the strut analysis, the mean TSL values were identical but the pQCT detected fewer nodes and free-ends than the histology.

The pQCT values of BV/TV, Tb.N, Tb.Sp correlated very well with the histological values ($r > 0.93$). The estimation of the mean Tb.Th of a given cylinder was fairly good ($r = 0.83$) knowing that the pixel size was 70 µm and the mean Tb.Th on histology was 84µm (Figure 30). In the strut analysis (Figure 31), the correlation for TSL was excellent ($r = 0.94$). On segmenting the skeleton, the pQCT scanner was less efficient to detect the Fe ($r = 0.79$) than the nodes ($r = 0.86$). The connectivity was better estimated by the pQCT when measuring the Star ($r = 0.91$, Figure 32) than the Nd/Fe ratio ($r = 0.81$).

Discussion

Density

The pQCT BMD was an excellent predictor of the true apparent density.

The physical properties of our samples are consistent with previously published results. We found a mean value 0.175 g/cm³ for the apparent density while Galante et al. (1970) found 0.19 g/cm³ and Hansson et al. (1980) 0.15 g/cm³, both of them using the weight of dry bone to estimate the apparent density.

Knowing a-priori the apparent density of a sample is most useful for biomechanical investigations (Keaveny et al. 1994) or when the sample itself has to be used for another purpose than density measurement (Bailey et al. 1999). However, for such non-destructive investigations to be useful, it should relieve the investigators from any processing of the sample before the measurement. First, if the marrow has to be removed and the sample to be processed before scanning, then why not directly weigh it? Second, for many biomechanical protocols, the bone marrow has to be retained in situ (Carter and Hayes 1977) (dynamic tests) and defatting is known to affect the mechanical properties (Linde and Sorensen 1993). Third, a non-destructive assessment of the density should be a fast procedure, not only to save time but also to avoid decay of the sample, especially if marrow is kept in place. With a scan time of less than 15 minutes, the cylinder was just thawed but still cold when going back to the deep-freezer. Consequently, the protocol was realistic, respecting the limitations of a density measurement preliminary to biomechanical tests.

However, there were two sources of random errors in our pQCT acquisition protocol. The amount of bone varies from the bottom to the top of the specimen (Banse et al. 2001). As the sample is not homogeneous, each cubic millimeter of the specimen should be analyzed by the pQCT to get as close as possible to the reference measurement that takes the whole specimen into account. The pQCT acquisition was limited to 4 slices, each 0.3mm thick and, consequently, we just scanned 2.5 ± 0.5 % of its total volume. This constitutes a first source of random error. Another source of inaccuracy was the variability of the

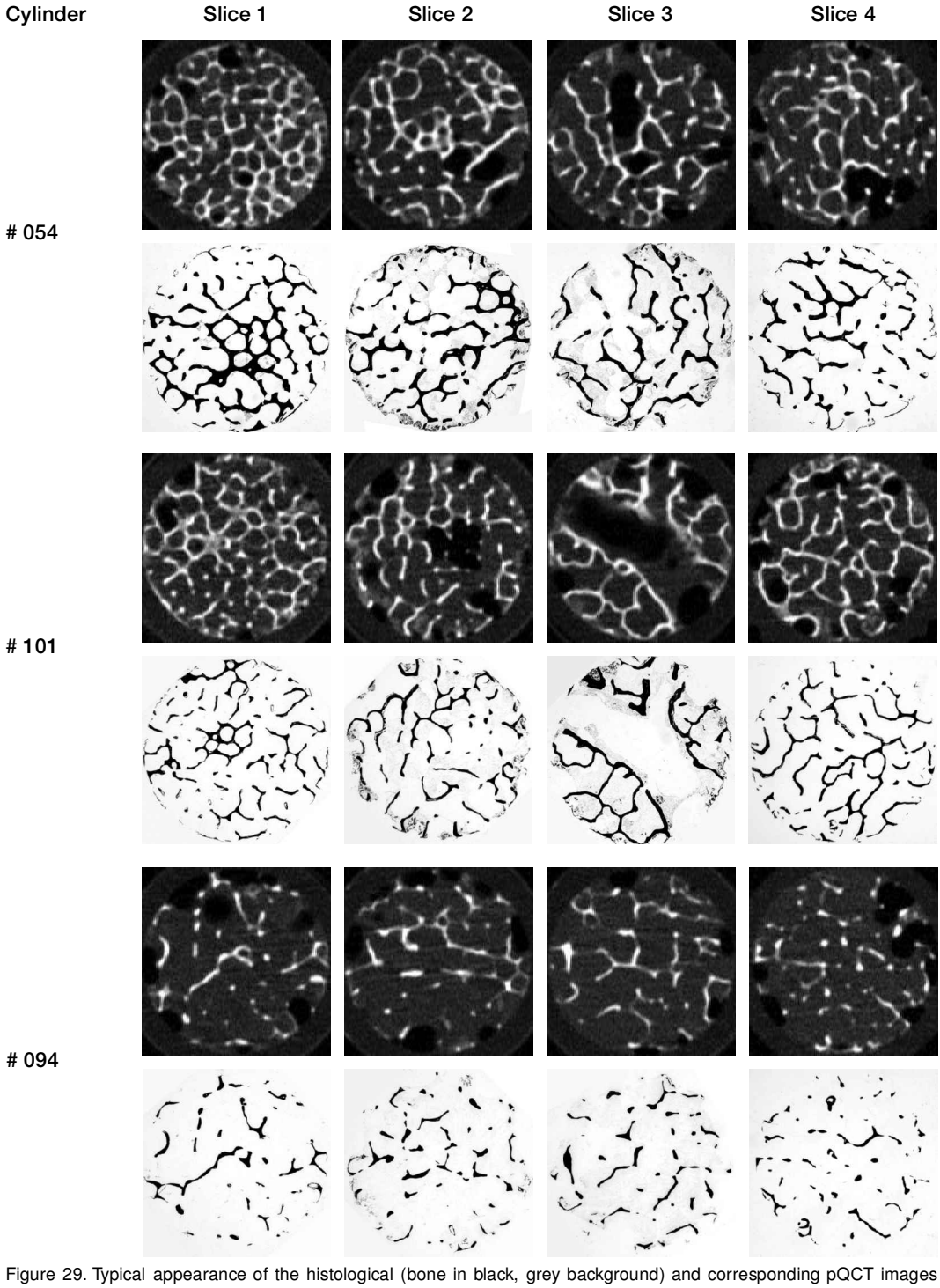

Figure 29. Typical appearance of the histological (bone in black, grey background) and corresponding pQCT images (bone in white, background black). Three cylindrical samples are shown. From the left to the right: Cylinder # 054 has a high density (BV/TV = 15.5%), relatively thick trabeculae, well connected. Cylinder # 101 has a medium density (BV/TV = 10.6%); note, in slice 3, that both techniques show the nice vascular channel crossing the cylinder. Cylinder # 094 illustrates a low density sample (BV/TV 5.4%) with a few very thin and disconnected trabeculae. (Modified from Banse et al. 2002b, with permission from Elsevier Science).

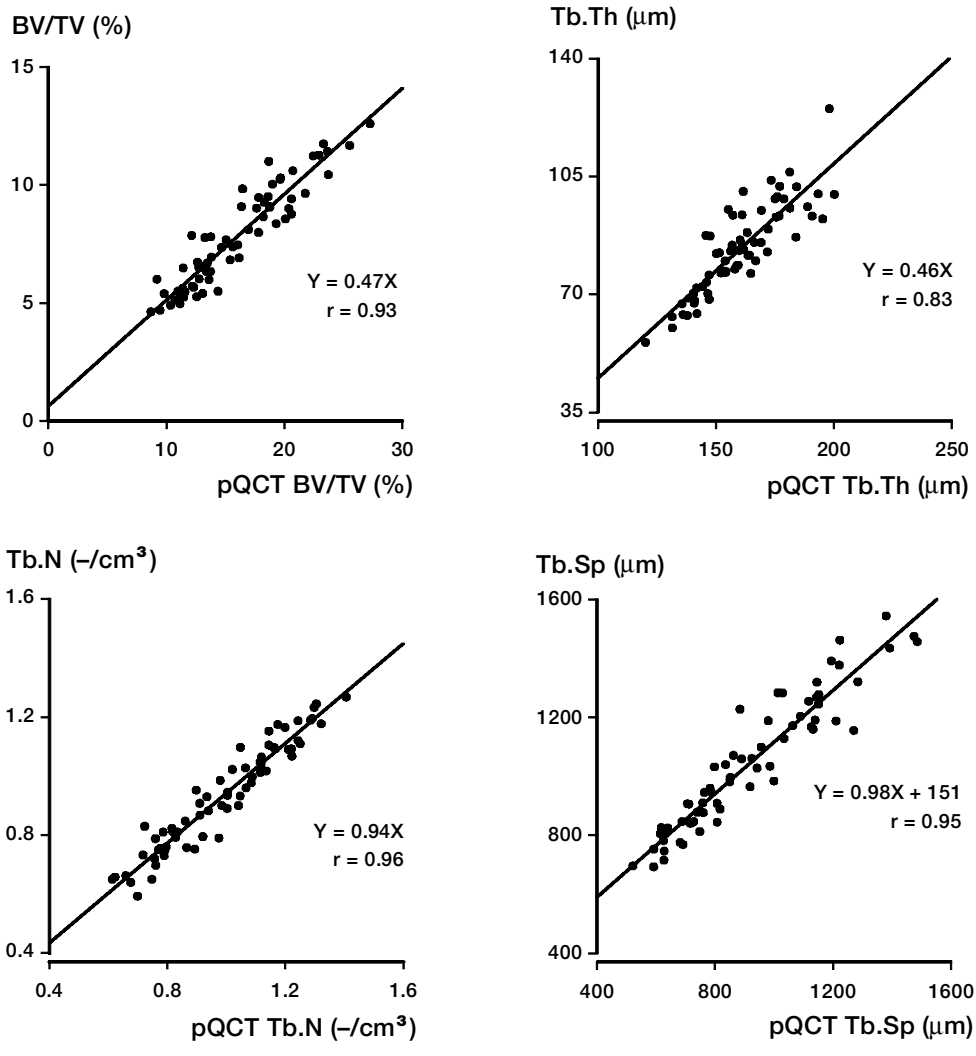

Figure 30. Diagram showing the correlation between the 'classical' morphometrical parameters obtained from histology and pQCT. They are all based on the detection of bone surfaces and perimeter. Even if the pQCT detects a bone surface twice as big as the histological control, the correlation is excellent.

nature of the medium surrounding the trabeculae. Air bubbles could certainly represent a problem, but also the more or less 'fatty' nature of the marrow (McBroom et al. 1985). The pQCT machine itself is calibrated in such a way that (when expressed in BMD value) the air value is –282 mgHA/cm³, the fat value is 0 mgHA/cm³ and the saline value (or the red marrow) is 50 to 60 mgHA/cm³. If all the pixels are considered, the mean BMD of a sample in air (with 15% of bone and 85% of air) is not the same as that of the same sample in water (15% bone and 85% water). The only way to eliminate this second source of inaccuracy is to remove everything (air and marrow) and to replace it by another homogeneous 'medium' (usually air, water or saline) (Jiang et al. 1998; Ebbesen et al. 1997). Fortunately, the pQCT has the capacity to eliminate most of this error with the image segmentation. A preliminary study (Banse and Devogelaer 2000) indicated that the optimal threshold was 200 mgHA/cm³. The thresholding procedure selected a surface equivalent to twice the bone volume fraction (Figure 30). For instance, when the bone

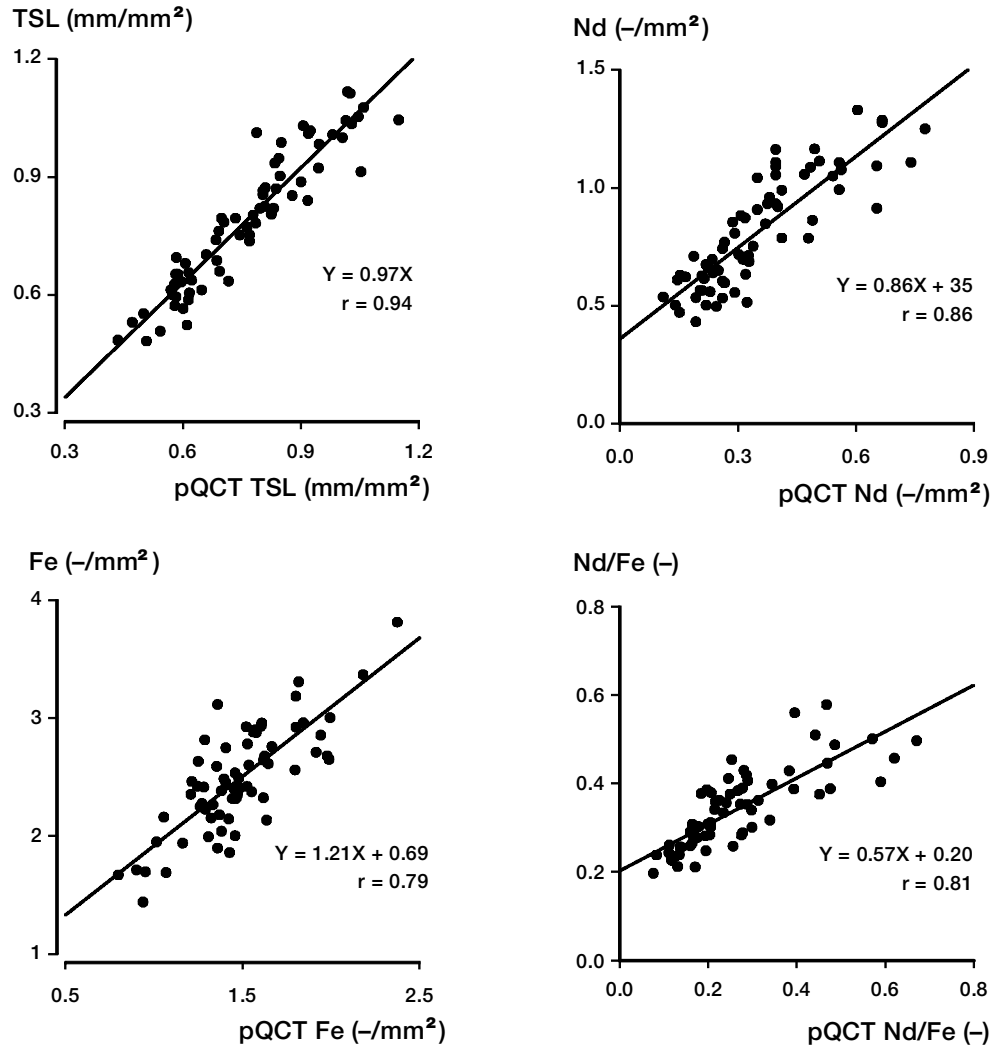

Figure 31. Performing a strut analysis on the pQCT images was also efficient. The total strut length (TSL) was identical on both types of image and the correlation was excellent. Detection of the Fe seemed less efficient with the pQCT.

volume fraction was 10%, the pQCT selected 20% of the pixels, thus eliminating the majority of the 'non bone pixels'. This thresholding procedure probably reduced the scattering effect or random error due to the marrow and air bubbles.

For the prediction of apparent ash density of 95 iliac crest samples (with bone marrow in place but no air bubbles) Ebbesen et al. (1997)obtained an identical correlation coefficient (r = 0.98).

These data validate the conversion of pQCT BMD values to apparent density as we did in Part I.

Morphometry

Practically, the pQCT gave a correct image of the vertebral bone morphology (Figure 29) and gave almost the same amount of information as the histology (Figure 30, 31 and 32). This is somewhat puzzling when comparing the volume of interest that was considered to give its gray value to each pixel. This volume is called voxel by the radiologists, and corresponds to the pixel size x the slice thickness. Basically this gray level tells us if we need to consider that precise volume as being bone or marrow. The voxel size with the pQCT was 1,470,000 μm³ (70 × 70 × 300 μm) and 500 μm³

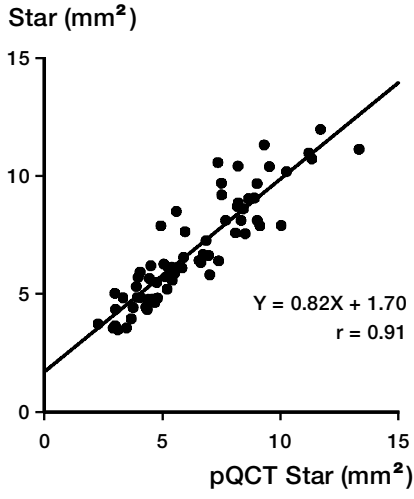

Figure 32. The pQCT correctly identified the samples with high Star, or those with relatively low connectivity.

($10 \times 10 \times 5$ µm) on the thin histological sections.

Three technical details have to be mentioned. First we did not make any effort to match the true BV/TV with the amount of selected pixels in the pQCT images. Our idea was that we preferred to overestimate BV/TV than to loose structural elements. Forcing the pQCT BV/TV to fit the true histological value (8%) has a price: a lot of structural elements are lost (Kothari et al. 1998; Ito et al. 1998). Secondly, the number of images (four) used for the assessment of a given cylinder is identical with both type of images. Morphological parameters from the pQCT were not extracted from a volumetric acquisition (Laib and Ruegsegger 1999; Muller et al. 1998). This means that the same surface of bone (in the images) is used to extract morphological parameters (Figure 29). Third, both type of images (Histological and pQCT) were treated by the same macro, with the same software and on the same computer, as to increase the comparability of the data.

Studies directly comparing scanner images with histological sections are scarce. Muller et al. (1998) obtained comparable correlation coefficients for the standard morphometrical parameters but their voxel size was 500 times smaller (2,744 µm³, 14 x 14 x 14 µm3) than the one used in this study. Classical morphometrical parameters were measured with a µCT on 63 *iliac crest* biopsies and yielded an almost identical correlation coefficient as found in this study (they reported r for BV/TV = 0.93, Tb.Th = 0.84, Tb.Sp = 0.91). Kuhn et al. (1990) obtained similar results but their threshold value was adapted to account for putative variation in the tissue density.

The only work comparing high resolution CT scanner and histo-morphometry *on vertebral bone* was carried out with samples coming from ewe (Mitton et al. 1998). In this species the BV/TV is much higher than in humans and their resolution was lower than ours (voxel size of 3,375,000 µm³ or $150 \times 150 \times 150$ µm). Consequently, they observed slightly lower correlation coefficient. However, the consistent point is that when running a strut analysis they also observed a good correlation with the TSL (r = 0.83) and experienced more difficulties to identify the Fe (r = 0.52).

The good correlations we observed between histology and pQCT may be linked to the structure of vertebral cancellous bone. The true BV/TV (measured by histology) is very low in the human vertebral body (90% of the total volume is occupied by marrow) and the space between trabeculae is pretty high (about 0.5 to 1.5 mm) allowing a correct identification of the trabeculae by pQCT.

With the simple threshold algorithm used for pQCT image segmentation a pixel was accounted as being bone, even when only a part of the voxel (50%) was truly occupied by bone. This explains the overestimation of the Tb.Th with low-resolution scanners as well as the two times higher BV/TV we observed. With the pQCT, every trabecula was considerably 'thickened' by the scanner (Figure 29 and 30B) but, provided that each feature (trabeculae) is sufficiently separated from the others, this phenomenon does not seem to prevent a correct morphometrical assessment.

On the pQCT images the identification of the multiple points or Nd from the skeleton was fairly good (r = 0.86) but the detection of the free ends (r = 0.79) seemed to be more problematic. This may be related to technical reasons. Slices were so thick (300 µm) with the pQCT that a bony feature disconnected on the histological image (5 µm thick) appeared as connected on the pQCT image. Considering a perforated trabecular plate (a frequent event), the histological image will show a disconnection, while a few microns below and above, the structure is actually connected with the rest

Table 9. η^2 **value for detection of between subject differences with pQCT and histology**

	Technique used	
Parameter	pQCT	Histology
Tb.Th* (µm)	0.64 [c]	0.73 [c]
Tb.N* (–/mm^2)	0.62 [c]	0.63 [c]
Tb.Sp* (µm)	0.55 [c]	0.62 [c]
TSL* (mm/mm^2)	0.61 [c]	0.58 [c]
Nd* (–/mm^2)	0.15	0.49 [c]
Fe (–/mm^2)	0.67 [c]	0.70 [c]
Nd/Fe* (–)	0.21	0.34 [b]
Star* (mm^2)	0.39 [c]	0.54 [c]

η^2 value is an estimation of the part of the overall variance that is due to differences between the subjects. P-value is for test that the effect of the considered variable = 0.
Key: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.0001$. * indicates that the BV/TV has been introduced as covariable.

of the network. We do not know which method gives mechanically relevant measurements. Technically, the pruning algorithm certainly influenced the number of Fe. For the analysis of both pQCT and histological images we removed all parasiting branches with a length less than the Tb.Th. However, while the length of the network was identical for both types of images, the Tb.Th was 84 µm on histology and 163 µm on pQCT images. Consequently we probably removed more parasiting branches with the pQCT. As such element is composed of one Nd and one Fe, this explains why we have less Nd and less Fe on the pQCT images.

Detection of differences between patients

A systematic analysis of the morphometrical parameters allowed us to pick up those that have the best ability to differentiate patients. Considering Table 9, it seems clear that Fe and Tb.Th are the most sensitive parameters. Interestingly, this holds true for both pQCT and histological analysis. For the detection of between-subjects differences, the pQCT is slightly less efficient than the histological analysis (η^2 values were lower for the pQCT). However, the estimated marginal means correlated very well in both methods (except for the nodes and node related parameters). When a clear patient specific structural pattern was detectable by the histology, the pQCT picked up the same information (i.e. this patient has more Fe, smaller trabeculae, higher Star….). The only parameter for which the pQCT lost the patient specific information was the Nd.

Confirmation

Overall, we consider the pQCT morphological information as reliable. In parts II and IV we used microarchitectural parameters determined with histology on 68 samples. What if we use the pQCT microarchitectural data trying to improve the prediction of the mechanical behavior (with n = 136) ? None of the pQCT morphometrical parameters significantly improved the prediction of bone stiffness and strength. Maybe the Tb.N slightly improved the prediction of bone strength (partial correlation coefficient r = 0.21, p = 0.015). However such an improvement was marginal.

In part IV the cross-link profile of a given cylinder was compared to the microarchitecture of the paired controlateral one. We assumed that the morphometry would be identical on both left and right sides. On comparing the pQCT data from both histology and biochemistry subgroups we obtained good correlation (r values between 0.75 and 0.9). Secondly, we can explore if the cross-link content of a cylinder is correlated with the pQCT microarchitecture of that same cylinder. Doing that, we clearly confirmed with the pQCT the observations reported in part IV: high pyrrole—or low HP—mean high pQCT Tb.Th (r = 0.59 and r = –0.50) and less pQCT Fe (r = –0.45 and r = 0.34).

pQCT as investigation tool

Overall, these data indicate that the pQCT is an excellent investigation tool to measure both bone density and microarchitecture. This was true even in poor conditions (with air bubbles, marrow and few slices).