Photoacoustic and ultrasound imaging of cancellous bone tissue

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1 Introduction

Bone is a complex three-dimensional (3-D) biostructure composed of both organic (mainly collagen Type I) and inorganic (hydroxyapatites) phases. The contributions of both parts are vital for bone integrity and proper functionality.1–3 Although the bone mineral density (BMD) is definitely a major factor, bone strength and integrity are affected by many other factors as well. The shape and microstructure of the bone tissue as well as the bone composition (minerals versus organic parts) have enormous influence on how bones thrive.4,5 While minerals can support compression stresses, collagen endures tensile stresses. Furthermore, collagen plays an important role in dissipating mechanical energy, which increases bone toughness and, therefore, reduces the risk of fracture.1 It is no wonder that even a minor change in collagen molecule architecture, for instance, due to genetic diseases, can induce huge macroscale defects in bone.1 Osteoporosis disease (OPD) is normally associated with a reduction in bone minerals, however, some research points to a correlation of fracture risk with changes in collagen content (CC) and collagen crosslinks.5–7 Only a few studies have investigated the bone organic phase variation during OPD8–10 and aging.11,12, more research in this field is highly desirable.

Despite the ionizing radiation, dual-energy-ray absorptiometry (DXA or DEXA) measurements of BMD represents the current gold standard for OPD diagnosis and fracture risk assessment.4 Quantitative ultrasound (QUIS) is another choice for assessment of bone health. The QUS operating principle is based on the measurement of the speed of sound (SOS) and the normalized broadband ultrasound attenuation (nBUA).13,14 Other methods based on the measurement of two waves or ultrasound (US) backscattering have also been proposed.5–7 US backscattering from bone depends on the mechanical properties and the microstructure of the interrogated hard tissue,17 which render it a good measure of bone health. US backscattering is normally represented by parameters such as backscatter coefficient,8,19 broadband ultrasound backscatter (BUB),20,21 integrated reflection coefficient,22 and apparent integrated backscatter (AIB).23–25 These parameters were extensively correlated with BMD or bone volume fraction (BV/TV) in the literature.26–28

Comparison between QUS images and BMD was also performed by Jenson et al.27 BMD was obtained from x-ray quantitative computed tomography (QCT) and three different QUS parameters: SOS, nBUA, and BUB were measured at 1 MHz on 38 human trabecular bone samples. The above-mentioned QUS parameters were calculated on 7 × 7 mm² regions of interest (ROI) on the surface images and were compared with the corresponding ROI in QCT. Significant correlation was found between QUS parameters and BMD where the transmission-measured parameters (SOS and nBUA) demonstrate stronger correlation with BMD than the backscatter-measured parameter (BUB). In contrast to the extensive research reported on the QUS response to BMD variation, only a few authors have studied the effect of bone CC variation on QUS parameters.29,30,31 It has been shown that SOS decreases and nBUA and AIB increase with decollagenization, although the correlations are weak.27

Recently, measurements of photoacoustic (PA) backpropagation from bones were reported for the first time.30,31 Since PA is sensitive to the optical properties of tissue, it can detect composition variation and as such it can provide complementary...
information to US backscattering measurements. In our previous
studies, we showed the sensitivity of PA with changes in min-
erals and organic phases of bone. We measured backpropa-
gating US and PA signals on identical locations of bone samples
before and after artificial demineralization and decollageniza-
tion. In the present study, we used both US and PA to
image the surface of trabecular bone samples in which half of
the bone had been treated. Thus, the obtained raster scanned
images were able to provide better insights of US and PA sen-
tivities to bone demineralization and decollagenization pro-
cesses. Then the images were compared with microcomputed
tomography (μCT) images of the samples, an independent well-
established diagnostic analysis tool used as the gold standard in
this context. In view of the very different spatial resolution
scales, in order to correlate the μCT and the US-PA measure-
ments, the μCT values were averaged over a rectangular cube.
The square surface of the cube was chosen to correspond to the
lateral resolution of the ultrasonic transducer, and the depth of
the rectangular cube was selected according to the time-gated
signal. The image comparison results confirmed the conclusions
provided by individual point measurements about the sensitivity
of US and PA modalities to demineralization and decollageni-
zation. The paper concludes with a discussion of the correla-
tion of both US and PA measurements with the BV/TV derived
from μCT.

2 Materials and Methods

2.1 Bone Specimen Preparation

Eight bone samples were harvested from the femurs of two cattle
(Angus, Canadian) and cut into blocks. The samples were
washed and then immersed and kept in saline solution for up
to 2 days to wash out the blood and marrow as much as possible.
The samples were then stored in a refrigerator before being
treated or measured and were allowed to equilibrate thermally
at room temperature prior to all experiments. The specimens
were separated into two groups and during the experiments,
they were treated with different agents to reduce their mineral
or CC. They were treated either with a 50% buffered
solution of ethylenediaminetetraacetic acid (EDTA) (pH = 7.7)
to demineralize the bone, or with a 5% solution of sodium liquid
hypochlorite to reduce the CC.

2.2 Experimental Setup

The experimental setup is shown schematically in Fig. 1. It was
designed to accommodate separate PA and US tests, as well as to
perform multiple measurements before and after treatment at the
same coordinate point of every sample.

Optical generation of PA signals was induced by a continu-
ous wave 805-nm diode laser (Laser Light Solutions, New
Jersey). The laser driver was controlled by a software function
generator for laser intensity modulation using a National
Instruments DAC card PXI-5421 (National Instruments, Texas).

For the US experiments, a 3.5-MHz transducer (V382
Olympus NDT Inc., Panametrics) was used as the transmitter of
modulated US. It was driven by a waveform generator identical
to that of the laser driver. No power amplifier was required to
boost the ultrasonic transmission due to the application of coded
excitation. A 2.25-MHz ultrasonic transducer (V305 Olympus
NDT Inc., Panametrics) was used as the receiver with a 40-dB
preamplifier (5676 Olympus NDT Inc., Panametrics) employed
before the ADC card.

Signal acquisition was performed through a National
Instruments PXI-5122 ADC card. To provide reference signals
that represent the dynamics of the instrumentation (i.e., function
generator, transducers, and preamp) regardless of the sample,
the spectra of US reflection signals from a polished metal
and PA backpropagating signal from a homogeneous absorber
were obtained. The reference US and PA spectra are shown in
Fig. 2 and were used later in the calculation of integrated US and
PA parameters. More details about the experimental setup and
signal analysis are described in our previous report.

The chirp duration was 1 ms for both PA and US probes.
However, different frequency sweeps were used for the two
modalities. The frequency range employed for the PA chirp was
300 kHz to 2.6 MHz and for the US chirp was 300 kHz to
4 MHz. The rationale for these frequency sweeps was to maxi-
mize the signal-to-noise ratio (SNR). The reference signals
(Fig. 2) show the sensitivities of the implemented PA and US
system versus frequency and justify the optimal frequency
range selected for each modality. The US transmitter element
was selected based on obtaining the same lateral resolution
in the focal point as the receiver transducer. Both transducers
had approximately identical focal zone widths of ~1 mm
(0.87 mm and 0.9 mm for transmitter and receiver,
respectively). Also, the size of the element was a critical factor. The diameter of the transmitter was 0.5 in., smaller than the 0.75 in. for the receiver. The center frequency was the second priority. Both of the transducers were wideband and covered the chirp frequency ranges. As a result, the sensitivity of the US measurements was reasonably good and it did not even require power amplification as mentioned before.

To perform accurate and fast raster scanning over the bone surface, a step motor and its DC servo controller (PT1-Z8 and TDC001, Thorlabs) were employed. The signal generation, acquisition, and servo controller were controlled by an in-house developed Labview program (National Instruments, Texas), which allowed for synchronization between signal generation and acquisition. The motor was used to scan the sample in steps of 1 mm. A total of 20 points were interrogated on each scanned line. Displacement in the other (vertical) direction was effected manually using a micrometer stage.

To identify the boundary between the treated and the intact parts of bones, two holes were drilled on their surface serving as landmarks. A screw was attached to the bone far from the measurement zone and was used to fix the bone to the stage for measurements and to allow for hanging the sample over the solution for partial immersion. The samples were immersed either in the demineralization or in the decollagenization solution so that the landmarks were located at the level of the solution–air interface, as shown in Fig. 3(b). These landmarks, as well as additional artificially made landmarks, were also used in identifying the exact points during μCT and for measurements at specific points on the surface of the bone before and after the various treatments [Fig. 3(c)].

μCT is a well-established bone assessment method, which has been used to validate the US and PA backpropagated signal correlation to BV/TV. These comparisons can highlight differences and the degree of complementarity between modalities. The samples were scanned using the commercial system (μCT 40-Scanco Medical AG, Brütisellen, Switzerland). For each bone, between 898 and 1933 μCT, signal slices were generated depending on the length of the bone. Virtual image slices were produced using an 18-μm stepsize with a 18-μm-pixel resolution at each slice. The slices were then converted to two-dimensional Digital Imaging and Communications in Medicine (DICOM) images, which were analyzed by an in-house developed MATLAB program. The MATLAB program first generates a 3-D matrix characterizing the 3-D bone model based on the μCT slices. Then the planes rotate in three directions to adjust the direction to the measured surface and the program adjusts the matrix accordingly. At this stage, it is possible to calculate the BV/TV at any subvolume of the matrix and compare it with measurements. The high resolution of μCT enables very accurate assessment of BV/TV in very small volumes. However, the lateral resolution of US and PA signals is limited to the lateral resolution of the transducers which, in our case, was ~1 mm. Our MATLAB program performs averaging over a volume of 1 mm × 1 mm (on the surface) × 4 mm (in depth). The time-gating process allows the extraction of the part of the signal corresponding to this depth and the square area corresponds to the lateral resolution of the detection system. Thus, the averaged BV/TV in this volume also corresponds to the measured US and PA signals.

Decollagenization was performed using sodium hypochlorite solutions (NaOCl) with 5% concentration and was applied for 3 h. EDTA 50% was used as a demineralization solution and was applied for 3 h. After the designated time, the sample was washed and measurements were performed again. The four samples which were demineralized with the EDTA solution will be henceforth labeled 1a, 2a, 3a, and 4a. The other four samples, which were decollagenized through treatment with the sodium hypochlorite solution, will be labeled 1b, 2b, 3b, and 4b.

It is informative to provide an evaluation of the extent of demineralization and decollagenization. Figure 4 shows the μCT cross-sectional images of samples 1a and 1b at 2 mm
beneath the surface after the treatments. Parts of these samples below the landmarks were demineralized and decollagenized, respectively. Arrows point to the landmarks on the surface of the samples.

As mentioned before, several factors besides minerals and CC can affect the PA and US signals. One important factor is the alignment and anisotropy of trabeculae. Since μCT measurements of the samples were available, assessment of the alignment and anisotropy was feasible. One quantitative and accurate method for assessing these factors is the use of a Gabor filter. The procedure is described elsewhere, and briefly explained here. The ROI is selected from a μCT slice 2-mm beneath the surface from each part of the bone samples: treated and intact. The Gabor filter was applied to the μCT slice by 10 deg rotational steps. Plotting the results in different directions explained here. The ROI is selected from a μCT slice 2-mm beneath the surface from each part of the bone samples: treated and intact. The Gabor filter was applied to the μCT slice by 10 deg rotational steps. Plotting the results in different directions

### Table 1
The extent of demineralization and decollagenization of samples, as well as their degree of anisotropy (DA).

<table>
<thead>
<tr>
<th></th>
<th>Extent of Demineralization [%]</th>
<th>Extent of Decollagenization [%]</th>
<th>DA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demineralized samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>−20.5</td>
<td>14.7</td>
<td>1.93</td>
</tr>
<tr>
<td>2a</td>
<td>−17.7</td>
<td>−7.8</td>
<td>1.33</td>
</tr>
<tr>
<td>3a</td>
<td>−30.5</td>
<td>13.1</td>
<td>2.8</td>
</tr>
<tr>
<td>4a</td>
<td>−16.6</td>
<td>7.1</td>
<td>2.10</td>
</tr>
<tr>
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<td>−21.9</td>
<td>6.8</td>
<td>2.04</td>
</tr>
<tr>
<td>Decollagenized samples</td>
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<td></td>
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<td>1.55</td>
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<td>−3.6</td>
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<td>1.82</td>
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<tr>
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<td>−6.5</td>
<td>−32.0</td>
<td>1.65</td>
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provides a directionality map of the μCT slice. Figure 5 shows four selected ROIs from samples 1a and 1b. Besides each μCT section, its calculated directionality map is also displayed. These directionality maps can help identify the main directionality in each part of the bones. Furthermore, they can also be used to calculate the degree of anisotropy (DA) of the trabeculae. The DA of all samples is reported in Table 1. The directionality and DA can affect the US and PA signals. The extent of the effect of directionality on the backscattered signal can be estimated from previous studies, where it was shown that the US backscattering coefficient is 1.8 dB lower in the anteroposterior (AP) than the mediolateral (ML) direction in human calcaneus at 500 kHz. Another in-vitro study showed that the US apparent integrated backscatter is less than 1 dB lower in AP and superoinferior directions compared with the ML in bovine tibia in the 1 to 3 MHz frequency range. These studies measured the US backscatter signal in completely different directions. In our study, the measurements were performed on two adjacent parts of the same samples; therefore, the effect of directionality was expected to be lower.

2.3 Experimental Verification of Water Absorption Effects on the Ultrasound and Photoacoustic Signals

For the sake of consistency in the results, we used a measurement method (protocol) similar in all experiments, which was also similar to our previous studies. We performed the measurements after letting the bone samples soak in saline solution for 2 h to be degassed. Therefore, it was important to investigate if water permeation had any significant effect on our PA and US measurements. We performed repetitive PA and US measurements at eight points on one sample (sample 1a) while it was immersed in saline solution. The sample remained in the solution for 10 h and the measurement was repeated every 2 h. Figure 6 shows the variation of PA and US signals at one location due to water immersion and soaking. It can be observed that the first peak changes (due to reflection) are only minor. The later peaks show some variation, however, the location and number of these peaks remain mostly unchanged. It should be noted that in the calculation of US AIB, the first peak is eliminated by time gating, but it is considered in the calculation of PA AIB. Figure 6(c) shows the average variation of the calculated AIB at 15 points during 10 h of immersion. The figure shows that the variation of PA and US AIB due to water absorption is much smaller than the variation due to position change on the bone surface. It should be mentioned that the samples were not dried after preparation and storage in the refrigerator. Therefore, the experimental results show the effect of changes following sample immersion in water for several hours.

2.4 Ultrasound and Photoacoustic Imaging of Bones

In the half-immersed sample geometry of Fig. 3, PA and US measurements were performed on the entire zone, as described in Sec. 2.2. Surface images are not only used for technique sensitivity assessment to bone decollagenization and demineralization, but also are very helpful for studying correlations with the corresponding μCT images. The comparison method is explained below. As shown in Fig. 3, several coordinate points on both sides of the boundary line landmarks were selected and measurements were performed before and after each treatment. Signal variance on the intact part of the bone reveals deviations due to factors other than decollagenization and/or demineralization.

Fig. 5 Selected areas of μCT sectional images of samples 1a and 1b (Fig. 4) and their corresponding directionality maps. These maps display the directionality and degree of anisotropy of the treated and intact parts of the bone samples.
The tested hypothesis was that other variations should be statistically insignificant compared to those incurred by decollagenization and/or demineralization. Eight or nine points on each half side of all samples were selected so that the distance between points, as well as between each point and the solution boundary, was at least 2 mm.

2.5 Image Analysis

Scanning experiments of US and PA back-propagating signals of all eight trabecular bone samples were performed. For proper comparison among the three measurement modalities (US, PA, and \( \mu \)CT), a quantitative analysis of PA and US experimental results was made as follows: AIB value images present the variation in the normalized signal for the treated and intact parts of the samples. The AIB was determined by frequency averaging (integrating) the ratio of the power spectrum of the time-gated signal \( P_b(f) \) to the power spectrum of a reference signal \( P_r(f) \) (Fig. 2) over the chirp frequency range:

\[
AIB = \frac{1}{\Delta f} \int_{\Delta f} 10 \log_{10} \left[ \frac{P_b(f)}{P_r(f)} \right] df.
\]  

(1)

Next, with regard to the US reference spectra (Fig. 2), it can be seen that the AIB calculation is weighed more toward the high frequency range of the signal spectrum than the middle range because of the division in Eq. (1). To normalize the frequency response, it is possible to apply a counter-weighing filter to AIB to offset the low components at the edges of the frequency range. However, since all of the signals in this investigation used the same set-up and laser/US intensity, the frequency-averaged power spectrum of the signal can be used for comparison. Similar to AIB, time gating was applied to extract the signal corresponding to backscattering down to a certain depth. Integrated-power images represent the overall variation of the backscattered signal power with and without treatment. To present the power in dB, normalization with the integrated-power spectrum of the reference signals (Fig. 2) was made and yielded the normalized apparent backscattered (NAB) signal, defined as

\[
NAB = 10 \log_{10} \left( \frac{\int_{\Delta f} P_b(f) df}{\int_{\Delta f} P_r(f) df} \right).
\]  

(2)

The values of AIB and NAB for both US and PA modalities were compared with the BV/TV from \( \mu \)CT. Also the correlations of these two modalities were compared with each other.
Depthwise images perpendicular to the boundary between intact and treated bone sectors presented some interesting features for comparison between PA and US modalities, which will be discussed below. The \( \mu \)CT image slices were averaged over the \( 1 \times 1 \times 4 \ mm^3 \) volume as described before from the same assessed surface of the bone. These images present the BV/TV within the volumes corresponding to the US and PA images. Image correlations reveal relative sensitivities of these modalities to BV/TV as well as signal dependencies on other factors.

### 3 Results

Figure 7 shows several images of the partly demineralized sample 1a. A photograph of the sample is shown in Fig. 7(a), with a box indicating the scanned area. Figure 7(b) is a \( \mu \)CT image generated by adding 223 slices corresponding to the 4-mm depth. The PA and US AIB images show the variation of PA backpropagation and US backscatter (AIB) within the scanned region, Figs. 7(c) and 7(d), respectively. Corresponding PA and US NAB images are shown in Figs. 7(e) and 7(f), respectively. The depth profile cross-sections generated by the PA and US

![Figure 7](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics)

**Fig. 7** Partially demineralized sample 1a: (a) photograph; (b) 4-mm collected \( \mu \)CT slices. Scanned images: (c) PA AIB; (d) US AIB; (e) PA normalized apparent backscattered (NAB); (f) US NAB; (g) PA image cross-section; and (h) US image cross-section. The location of cross-section images corresponds to the line box shown in (e) and (f).
cross-correlation functions are presented in Figs. 7(g) and 7(h), respectively; they show the signal attenuation profiles with delay time. It should be noticed that delay time corresponds to depth. The surface coordinates of the presented cross-sections are shown as thin horizontal bars in the NAB images, Figs. 7(e) and 7(f). The vertical line in the middle of each image represents the abovementioned landmark line, which was used to distinguish treated and intact sample halves.

Figure 8 is the counterpart of Fig. 7 with respect to sample 1b, which was partly decollagenized and they appear in the same order as Fig. 7. Figure 9 is a histogram of the surface-averaged PA and US AIB variations produced by point measurements on the treated and the intact parts of the bone samples before and after treatment. These variations show the sensitivity of each modality to decollagenization and demineralization. Figure 9 shows the reproducibility of the experiments, where the points in the intact part of the bone show very small variations.

4 Discussion

Figure 7 presents the surface scanned images of the partly demineralized sample 1a. It can be seen that the PA and US AIB and NAB exhibit significant changes in the demineralized half compared with the intact half of the bone. Similarly, in the depth profiling images, the reduction of both PA and US signals is evident. In this study, PA and US AIB were both found to significantly decrease with BV/TV reduction. This result is
consistent with previous reports where both modalities show sensitivity to changes in BV/TV of trabecular bone with US having higher sensitivity.\footnote{11,33}

Figure 8 presents the AIB and NAB scanned images of the partly decollagenized sample 1b. It is observed that the PA AIB decreased significantly after decollagenization, whereas the US AIB increased slightly. The consistency of these results with the other tested samples is addressed in the discussion of Table 2, where the average change of parameters for each sample and the total average over all samples are presented. The depthwise images also show a clear reduction of the PA signal in the decollagenized part of the sample, Fig. 8(g).

In Table 1, we show the extent of demineralization and decollagenization of the samples. The average differences of BV/TV of the treated and intact parts for demineralized and decollagenized samples are 21.9\% and 6.5\%, respectively. Furthermore, the average difference of CC of treated and intact parts for decollagenized samples is 32\% less and for demineralized samples is 6.8\% higher. These differences are reasonable and agree with the agents employed for treatment of the bone samples. Additionally, they support our conclusions on the effect of demineralization and decollagenization on US backscattering and PA backpropagating signals. The average differences of AIB and NAB parameters between the treated and intact parts of all bone samples are presented in Table 2. This table also shows the averaged BV/TV of the ROIs in the treated and intact parts of the bone samples.

The AIB and NAB values for all samples (Table 2) as well as the average changes (Table 1) show the consistency of the foregoing results for samples 1a and 1b with the remaining samples: both PA and US parameters (AIB and NAB) were reduced significantly in the demineralized part of the bone. PA signals were reduced significantly in the decollagenized part of the bone, while US parameters increased slightly in the decollagenized part. The behavior of US backscatter parameters is consistent with the QUS literature, where the slight increase of US backscattering with decollagenization is interpreted to be due to the decrease in acoustic wave attenuation.\footnote{28} Table 2 also reports the correlation coefficient between the PA and US parameters. It can be seen that PA and US parameters exhibit weak-to-moderate correlations for the demineralized samples, as they both respond fairly similarly to demineralization treatment. The NAB parameter shows stronger correlation between the two modalities. As discussed later on, the NAB parameter also correlates better with BV/TV. On the contrary, the PA and US parameters exhibit no correlation for the decollagenized samples. This can be understood by considering the different responses to reduction of CC by the two modalities: the US backscattering is affected by a decrease in attenuation, whereas the PA backpropagation is affected by the reduction in chromophore (absorber) density.

**Table 2** The correlation coefficient between US and PA parameters as well as difference between the AIB and NAB and bone volume fraction in treated and intact parts of the bones.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Difference between treated and intact bone [dB]</th>
<th>US-PA correlation</th>
<th>BV/TV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>US AIB PA AIB US NAB PA NAB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Demineralized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>−4.96 ± 3.36 −2.86 ± 2.54 −5.99 ± 4.02 −6.17 ± 2.68</td>
<td>0.250 0.206</td>
<td>0.215 0.171</td>
</tr>
<tr>
<td>2a</td>
<td>−2.28 ± 1.56 −2.29 ± 2.02 −4.84 ± 3.19 −2.43 ± 1.84</td>
<td>0.333 0.525</td>
<td>0.254 0.209</td>
</tr>
<tr>
<td>3a</td>
<td>−2.61 ± 1.24 −4.38 ± 3.53 −6.16 ± 3.03 −6.92 ± 1.86</td>
<td>0.358 0.561</td>
<td>0.269 0.187</td>
</tr>
<tr>
<td>4a</td>
<td>−3.31 ± 2.52 −3.78 ± 3.27 −3.59 ± 2.83 −4.02 ± 3.15</td>
<td>0.361 0.436</td>
<td>0.193 0.161</td>
</tr>
<tr>
<td>Average</td>
<td>−3.29 ± 2.32 −3.33 ± 2.9 −5.15 ± 3.3 −4.88 ± 2.45</td>
<td>0.326 0.432</td>
<td>0.233 0.182</td>
</tr>
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<td>Decollagenized</td>
<td></td>
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<td></td>
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<tr>
<td>samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>1.63 ± 1.92 −5.24 ± 1.73 3.74 ± 3.89 −5.81 ± 1.77</td>
<td>−0.405 −0.043</td>
<td>0.234 0.216</td>
</tr>
<tr>
<td>2b</td>
<td>1.02 ± 1.49 −3.04 ± 2.97 0.22 ± 2.05 −5.64 ± 2.82</td>
<td>−0.318 −0.126</td>
<td>0.183 0.175</td>
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<tr>
<td>3b</td>
<td>1.92 ± 1.32 −3.61 ± 2.25 2.81 ± 1.96 −4.22 ± 1.54</td>
<td>−0.584 −0.165</td>
<td>0.221 0.201</td>
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<tr>
<td>4b</td>
<td>1.40 ± 1.99 −6.27 ± 1.95 0.52 ± 2.12 −6.23 ± 2.08</td>
<td>−0.344 −0.143</td>
<td>0.165 0.159</td>
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<tr>
<td>Average</td>
<td>1.49 ± 1.7 −4.54 ± 2.27 1.82 ± 2.63 −5.48 ± 2.11</td>
<td>−0.413 −0.119</td>
<td>0.201 0.188</td>
</tr>
</tbody>
</table>

Fig. 9 PA and US AIB value variation histogram before and after treatment for intact and treated parts of the bone samples.
Table 3 The correlation coefficient between US or PA NAB and microcomputed tomography ($\mu$CT) in the treated and intact parts of the samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Intact part US/$\mu$CT</th>
<th>Intact part PA/$\mu$CT</th>
<th>Treated part US/$\mu$CT</th>
<th>Treated part PA/$\mu$CT</th>
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</thead>
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<tr>
<td>Demineralized</td>
<td>0.532 0.325</td>
<td>0.437 0.148</td>
<td>1a</td>
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<tr>
<td></td>
<td>0.757 0.388</td>
<td>0.532 −0.021</td>
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<td></td>
<td>0.743 0.554</td>
<td>0.353 0.199</td>
<td>3a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.683 0.371</td>
<td>0.469 0.162</td>
<td>4a</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.679 0.409</td>
<td>0.448 0.122</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decollagenized</td>
<td>0.756 0.392</td>
<td>0.821 −0.264</td>
<td>1b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.680 0.397</td>
<td>0.712 −0.309</td>
<td>2b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.607 −0.502</td>
<td>0.698 −0.189</td>
<td>3b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.548 0.287</td>
<td>0.473 −0.086</td>
<td>4b</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.648 0.144</td>
<td>0.676 −0.212</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The correlation coefficients between US or PA NAB and BV/TV (derived from the $\mu$CT images) in the treated and intact parts of the samples were calculated separately and are shown in Table 3. A moderate linear correlation between US NAB and BV/TV was found for the intact parts of all samples, with $r$ values from 0.532 to 0.757. A weak correlation between PA NAB and BV/TV was found for the intact parts of all samples. The correlation coefficient between US NAB and BV/TV slightly decreased after demineralization. On the contrary, the correlation between US NAB and BV/TV slightly increased after decollagenization. This can be understood by considering that the main effect of the organic parts of the bone on the US signal is attenuation, so that by decreasing the volume fraction of minerals, the US signal attenuation is affected the most. On the other hand, by decreasing the CC, the attenuation component due to the collagen reduces and the US signal correlation with mineral content is expected to increase. The correlation of PA NAB and BV/TV decreased after both treatments. Similarly, the correlation coefficients between US or PA AIB and BV/TV in the treated and intact parts of the samples are calculated and reported in Table 4. The correlation between the US AIB and BV/TV is weak-to-moderate for the intact parts of all samples, with $r$ values from 0.309 to 0.632, which are relatively small compared with those between the US NAB and BV/TV. Also, the PA AIB shows weak correlation with BV/TV after either treatment.

Table 5 The correlation coefficient between $\mu$CT and PA/US AIB including both treated and intact parts of the samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>US/$\mu$CT AIB</th>
<th>PA/$\mu$CT AIB</th>
<th>US/$\mu$CT NAB</th>
<th>PA/$\mu$CT NAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demineralized</td>
<td>0.263 0.324</td>
<td>0.514 0.421</td>
<td>0.514 0.421</td>
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<tr>
<td></td>
<td>0.442 −0.353</td>
<td>0.579 −0.433</td>
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<td></td>
<td>0.382 0.546</td>
<td>0.751 0.607</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.513 0.289</td>
<td>0.631 0.348</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.400 0.201</td>
<td>0.619 0.236</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decollagenized</td>
<td>0.358 0.312</td>
<td>0.724 0.370</td>
<td>0.724 0.370</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−0.373 0.658</td>
<td>−0.448 0.683</td>
<td>−0.448 0.683</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.342 −0.255</td>
<td>0.698 −0.378</td>
<td>0.698 −0.378</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.424 0.187</td>
<td>0.592 0.225</td>
<td>0.592 0.225</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.188 0.226</td>
<td>0.391 0.225</td>
<td>0.391 0.225</td>
<td></td>
</tr>
</tbody>
</table>
backscatter parameters with BV/TV. They are more effective at frequencies above 1 MHz where ultrasonic wavelength is on the order of trabecular dimensions, therefore, a lower correlation should be expected in comparison with similar studies, such as Jenson et al.,27 who used 1 MHz for their studies. For PA parameters, in addition to those factors, the molecular composition of the sample should also be considered; thus, it would be logical to expect weaker correlation for PA with BV/TV than for US signals.

The averaged correlation coefficients show that NAB provides better correlation than AIB with μCT and it is consistent for all cases of demineralized samples for both PA and US modalities. It should be noticed that in the NAB calculation, no spectral weighing was applied in postprocessing. Spectra are weighed automatically with the transducer transfer function, the PA energy conversion, and other elements of the transfer function involving the instrumentation. Therefore, noise does not increase in postprocessing. In the calculation of AIB, normalization with the reference signal spectrum applies a weighing function that enhances signal components at the frequency margins of the spectra where the SNR is small, thereby boosting noise. Additionally, the NAB contains more low-frequency content compared with the AIB due to the lack of the aforementioned artificial weighing, as can be seen in the reference spectra. These two factors justify the fact that NAB is a better metric for evaluation of US and PA backpropagation signals.

Figure 9 shows the average changes of PA and US AIB with demineralization and decollagenization. The filled columns show the changes of this parameter in intact bone in measurements before and after exposure to the solution. Thus, the filled columns define the variation baseline of PA and US AIBs due to changes in consecutive measurements without any treatment of the bone tissue. These “before” and “after” treatment results are similar to our previous studies32 and are consistent with the images presented in Figs. 7 and 8 for treated and intact parts of the bone.

The main purpose of this study was to examine the sensitivity of PA and US to variations in mineral and CC of the trabecular bone. Employing surface imaging on treated versus intact parts of bone samples provides a better understanding of the bone condition compared with random single-point measurements. In the presented experiments, single-element transducers were employed to maximize sensitivity. It is beneficial to use a customized designed array transducer matching the skeletal site under study for fast clinical application. However, a prerequisite to that stage is to establish AIB and NAB thresholds for reliable osteoporosis diagnosis.

One limitation of this study is that all the measurements were performed in vitro. Therefore, it is unknown how in vivo tissue and cortical bone overayers, as well as the presence of marrow, can affect the results. However, our previous experiments demonstrate the possibility of PA signal detection under a 1.5-mm-thick cortical layer.31 Similarly, other authors also reported the possibility of PA imaging under the skull layer.43,44 To mitigate errors due to the presence of soft tissue and cortical layer in QUS, dual-frequency ultrasound has been suggested.45,46 In those applications, measurements at two different frequencies were used to enable sufficient precision despite the existence of those overayers. With the PA modality, one may not only use different frequency ranges but also multiple wavelengths, which is a major capability when it comes to increasing accuracy.

5 Conclusions
In this study, we introduced a volumetric correspondence between the US backscattered and PA backpropagation parameters, and the μCT calculated BV/TV. The resolution of all modalities was numerically adjusted so that it would be similar for all three modalities. We also defined a modification for the AIB in QUS, which we named as NAB. Similarly, this parameter was also defined for PA detection and we showed that for wideband detection, it correlated with BV/TV most closely. This is due to omitting the synthetic weighting of the spectra, which sacrifices SNR. It may not be very important for limited bandwidth detection, but the effect was crucial for wideband detection.

In conclusion, our findings provide insights into PA and US modalities in which PA and US AIB and NAB values were found to decrease with demineralization (decreases in BV/TV), but they exhibit opposite trends with changes in CC, with PA signals being more sensitive to those changes: US signals increase and PA signals decrease with decreasing CC. The findings about US backscattering with demineralization and decollagenization are consistent with the QUS literature. As mentioned before, there exists a large body of literature on the US backscattering relationship with BMD,17,23,24,26,27 while only few studies have been reported on its relation to CC.28,29,21 In the QUS literature, the relation between US backscattering and CC is attributed to acoustic attenuation. PA backpropagation analysis is novel and reports in the literature are limited to this and our previous studies. From the present studies, it is concluded that PA and US raster scan images over portions of bovine bone samples exhibit weak and moderate correlations with BV/TV, respectively.

Considering that the prevailing OPD diagnostic methods, such as DEXA, are only BMD specific, a combination of PA and US probes may result in improved sensitivity and specificity for the detection of bone demineralization or decollagenization, leading to more accurate bone loss diagnosis.

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