Regular chondrocyte spacing is a potential cause for coherent ultrasound backscatter in human articular cartilage

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The potential of quantitative ultrasound (QUS) to assess the regular cellular spacing in the superficial cartilage zones was investigated experimentally and numerically. Nine osteochondral samples, extracted from two human cadaver knee joints, were measured using a 50-MHz ultrasound scanning device and evaluated using Mankin score. Simulated backscattered power spectra from models with an idealized cell alignment exhibited a pronounced frequency peak. From the peak, cell spacing in the range between 15 and 40 μm between cell layers was detected with an average error of 0.2 μm . The mean QUS-based cell spacing was $28.3 \pm 5.3 \mu\text{m}$. Strong correlation ($R^2 = 0.59$, $p \leq 0.001$) between spacing estimates from light microscopy (LM) and QUS was found for samples with Mankin score ≤ 3 . For higher scores, QUS-based spacing was significantly higher ($p \leq 0.05$) compared to LM-based spacing. QUS-based spacing estimates together with other QUS parameters may serve as future biomarkers for detecting early signs of osteoarthritis.

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I. INTRODUCTION

Load bearing function and nearly frictionless motion of joints are achieved by the unique combination of highly organized structure and material properties of articular cartilage (AC) and synovial fluid (Buckwalter *et al.*, 2005; Buckwalter and Mankin, 1998; Mow *et al.*, 1992). Osteoarthritis (OA) is a joint disease degenerating AC structure. Early signs of OA include a loss of proteoglycans and

subsequent softening of the cartilage matrix, particularly in the superficial zone, in which collagen fibrils are oriented parallel to the surface. Degradation of collagen network causes swelling of the tissue and weakens the tissue tensile properties (Buckwalter *et al.*, 2005). Chondrocytes, i.e., cartilage cells, are embedded into the AC matrix. In mature humans, these cells have a characteristic depth-dependent shape and spatial arrangement. In the superficial zone, small ellipsoidal cells are separated by thin sheets of collagen (Jadin *et al.*, 2005; Rolauffs *et al.*, 2008). In the deep zone, spherically shaped cells are larger in size and sparsely distributed in vertical columns (Hunziker *et al.*, 2002). During OA progression, cellular morphology undergoes remarkable changes. First, chondrocytes proliferate and clone in the superficial zone in an attempt to repair the damaged tissue by synthesizing cartilage matrix. Second, this phase of

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hypercellularity is followed by a phase of hypocellularity. At this point cartilage tissue is usually already damaged and cells undergo apoptosis (Thomas *et al.*, 2007) and the articular surface becomes fibrillated as a consequence of physical wear (Mankin and Lippiello, 1970). Therefore, if OA could be detected in the early stage of the disease, further progression may be prevented by conservative therapy approaches, e.g., by decreasing the risk factors such as obesity and muscle weakness. As changes in cell number, size, and distribution in the superficial cartilage zone are associated with the early progression of OA, they may provide a measure for the detection and grading of OA at the early stage of the disease.

Currently, none of the established non-invasive imaging modalities, such as native x ray, computed tomography, external ultrasound, or magnetic resonance imaging (MRI) are able to assess the OA-related degenerative tissue alterations in cell organization and morphology (Gold *et al.*, 2009; Liukkonen *et al.*, 2014; Nieminen *et al.*, 2009). MRI studies demonstrated its ability to grade and quantify morphologic changes and changes in the extracellular matrix (Li and Majumdar, 2013). However, high cost, lack of accessibility, and long examination times limit screening and treatment-monitoring capabilities of MRI. High-frequency ultrasound is a non-invasive, affordable, and accessible technology. By means of spectral analysis, quantitative information from subwavelength structures, e.g., cells, can be obtained, which could have a strong clinical impact in OA management.

Previous studies have demonstrated that high-frequency quantitative ultrasound (QUS) with frequencies above 20 MHz holds promise for the quantitative assessment of OA (Männicke *et al.*, 2014a; Schöne *et al.*, 2013). It enables the visualization of both the articular surface integrity (Aula *et al.*, 2010; Liukkonen *et al.*, 2014; Männicke *et al.*, 2014a; Schöne *et al.*, 2013) and the cartilage bone interface (Aula *et al.*, 2010; Liukkonen *et al.*, 2014). In addition to the examination of the cartilage interfaces, several studies have focused on the analysis of ultrasound backscatter from the cartilage matrix, which provides information about its structure (Chérin *et al.*, 1998; Männicke *et al.*, 2014a). The most commonly used parameter is the apparent integrated backscatter (AIB), which is the integrated backscattered acoustic energy over the bandwidth of the used transducer (Chérin *et al.*, 1998), either at a fixed tissue depth or in a depth-dependent way by shifting the time window of the analyzed backscatter signal (Männicke *et al.*, 2014a). Since its introduction AIB has been suggested to be sensitive to matrix composition and cellular structure (Inkinen *et al.*, 2014; Männicke *et al.*, 2014a) and empirical relations to cartilage degeneration have been shown (Männicke *et al.*, 2014a; Virén *et al.*, 2010). Recently, Männicke *et al.* reported that the Mankin score (Mankin and Lippiello, 1970), describing the state of cartilage degeneration by means of histological grading, can be predicted using regression analysis with different combinations of ultrasound parameter pairs (Männicke *et al.*, 2014b). While AIB was most predictive in advanced degeneration stages, the apparent frequency dependence of the backscatter amplitude (AFB) appeared to be predictive for the early stages of degeneration (Männicke *et al.*, 2014b). In a recent systematic study in different

species (human, ovine, and bovine), 51% of AIB variations could be explained by a combination of collagen concentration and cell number density (CND; Männicke *et al.*, 2016). However, the individual contributions of cells and extracellular matrix to the ultrasound backscatter properties of AC are still poorly understood. Complementary to these mainly empirical studies, modeling cartilage tissue paired with numerical ultrasound simulations could provide a better understanding of the complex backscatter processes and could lead to refinement of QUS-based OA classification.

A study by Rolauffs *et al.* (2008) provided evidence that chondrocytes in the superficial zones of hyaline cartilage are organized in sheets of cell clusters pervaded by collagen fibers, which are aligned parallel to the articular surface. We hypothesize that the cell clusters are stratified with a certain degree of regularity in healthy hyaline cartilage causing a contribution of coherent backscattering, in addition to the incoherent backscatter, when interrogated in the appropriate frequency range. Moreover, we hypothesize that if such layered structure is present, it should give rise to a coherence peak potentially detectable in the power spectra of the ultrasound signals backscattered from cartilage. The objective of this study was to investigate whether a peak in the frequency spectrum is observable in clinically relevant frequency bandwidth and if apparent, whether it can be explained by a layered organization of chondrocytes. In addition, conventional QUS parameters AIB and AFB were determined. To test our hypothesis, we conducted 50-MHz backscatter measurements *ex vivo* on nine human osteochondral samples. Moreover, numerical ultrasound propagation models were developed to confirm the appearance of resonance peaks and to develop appropriate signal processing methods to estimate the cell spacing from these peaks. Characteristic cell morphology and material properties of the human cartilage tissue models were assessed from light microscopy (LM) and 250-MHz acoustic microscopy, respectively. In contrast to simpler models of confluent scatterers distributed in layered media (Couture *et al.*, 2007; Franceschini *et al.*, 2014), this approach allowed the investigation of the effects of the complex cartilage architecture, i.e., low cell and cell-layer numbers, depth-dependent changes of cell shape and spacing, non-confluent cell layers pervaded by sheets of collagen layers, and the incorporation of realistic material properties.

II. MATERIALS AND METHODS

A. Sample collection and preparation

Osteochondral samples ($N = 9$) with visually intact cartilage surfaces or mild signs of OA were collected from left and right knee joints of two human cadavers [male, 67 yr, $N = 4$; female, 51 yr, $N = 5$; Fig. 1(A)]. Osteochondral samples (diameter, 10 mm) were extracted from the lateral femoral condyle ($N = 3$), medial tibia plateau ($N = 3$), and upper quadrant of the patella [$N = 3$; Fig. 1(A)]. In order to prevent degradation, samples were stored in phosphate buffered saline at 4 °C including inhibitors of proteolytic enzymes (5 mM ethylenediaminetetraacetic acid disodium salt, EDTA, VWR International, Fontenay, France; and

FIG. 1. (A) Illustration of the anatomical locations where osteochondral samples were collected, as well as the abbreviations for the locations: lateral femoral condyle (LFC), medial tibial plateau (MTP), and lateral upper quadrant of patella (LP). (B) First, the osteochondral plug was scanned using 50-MHz QUS in x and y directions ($40\text{-}\mu\text{m}$ step size) with the beam focus $\sim 500\text{ }\mu\text{m}$ below sample surface. Subsequently, the plug was cut in half and from the halves $10\text{-}\mu\text{m}$ and $5\text{-}\mu\text{m}$ thick sections were prepared for 250-MHz scanning acoustic microscope (SAM) and histology, respectively.

5 mM benzamidine hydrochloride hydrate, Sigma Aldrich Inc., St. Louis, MO), antibiotics (Penicillin streptomycin, 100 units ml^{-1} penicillin, $100\text{ }\mu\text{g ml}^{-1}$ streptomycin; EuroClone, Sizzano, Italy), and an antimycotic agent (Gibco Fungizone Antimycotic, $250\text{ }\mu\text{g ml}^{-1}$ amphotericin B, $205\text{ }\mu\text{g ml}^{-1}$ sodium deoxycholate; Life technologies, Carlsbad, CA). Kuopio University Hospital ethical committee reviewed the study protocol, including experiments on human samples (favorable opinion: 58/2013).

The osteochondral plugs were first interrogated using 50-MHz QUS to collect backscatter signals with the sound propagation direction perpendicular to the cartilage surface [depth-direction; see Fig. 1(B)]. Then, the samples were fixed in 4% paraformaldehyde and embedded in paraffin. Subchondral bone was removed from the specimens and $10\text{-}\mu\text{m}$ thick cross sections were cut and transferred to a glass microscopy slide for the 250-MHz scanning acoustic microscope (SAM) measurements and adjacent $5\text{-}\mu\text{m}$ sections were prepared for histology.

B. LM-based cell segmentation and Mankin score

Histology sections were stained with Safranin-O and imaged with a light microscope (Zeiss Axioimager M2, Carl Zeiss Microscopy GmbH, Jena, Germany) to evaluate the cell morphology and degeneration grade of the samples [Fig.

FIG. 2. (Color online) (A) A Safranin-O stained section of the sample, and (B) SAM amplitude image of a cartilage section from the same specimen but from different locations (adjacent section). Region of interest (ROI) is marked as a square in the center and arrow points to a detached surface area. From the same section, corresponding bulk modulus (C) and mass density (D) maps are presented. For presentation purposes, the parameter maps were de-noised using a square 2×2 pixel (i.e., $16\text{ }\mu\text{m}^2$) median filter.

2(A)]. The latter was done using the Mankin scoring method (Mankin and Lippiello, 1970). Three sections from each sample were scored independently by three scorers and the results were averaged to nearest integer. Samples were divided into two groups based on the Mankin sub-score I, which scores the structural integrity. Group I consisted of samples with Mankin subscore $I < 2$, in which the superficial zone was still completely or almost intact. In this group, the total Mankin score was ≤ 3 . Group II consisted of samples with Mankin subscore $I \geq 2$. In this group, the total Mankin score was 5 or 6.

Cell size for the numerical simulations was evaluated from manually segmented cells located in a depth range up to $250\text{ }\mu\text{m}$ below the cartilage surface. To estimate the cell spacing based on the LM measurements, each segmented image was divided into overlapping regions of interest (ROIs) (depth, $100\text{ }\mu\text{m}$; width, $500\text{ }\mu\text{m}$). The overlap in both directions was 90%. Each vertical line within the ROI was gated using a Tukey window and the power spectrum was calculated. Then, the spectra from all horizontal positions within the ROI were averaged, converted to logarithmic scale, and the position of the maximum was determined. This peak frequency f_{peak} was converted into a spacing value using the relation d_z/f_{peak} , where d_z is the pixel size in the vertical direction. For comparison with the US-based spacing estimates, the spacing values were averaged in $25\text{-}\mu\text{m}$ intervals between $100\text{-}\mu\text{m}$ and $250\text{-}\mu\text{m}$ depths relative to the cartilage surface.

C. Numerical ultrasound propagation simulation setup

Two-dimensional (2D) ultrasound wave propagation simulations were conducted using SimSonic.¹ The code is based

FIG. 3. (Color online) (A) Model geometry showing transducer array, coupling medium (black), and cartilage. The latter consists of two layers with distinct cellular structures. A zoomed region from the red box in (A) is shown in (C). A virtual unfocused transducer array is centered 2 mm above the cartilage surface for transmission of a plane wave and reception of signals. (B) Snapshot of the transmitted, reflected, and backscattered waves after $2.2 \mu\text{s}$ propagation time.

on a finite-difference time-domain (FDTD) scheme and was developed by [Bossy et al. \(2004\)](#). The simulation setup was designed to investigate the effect of idealized spatial cell distributions on backscattered signals under well-controlled conditions. The FDTD method accounts for complex phenomena, e.g., irregular and heterogeneous structures, multiple scattering, wave mode conversions, attenuation, refraction, or diffraction. Our model geometry consists of cartilage matrix with embedded cellular structure and immersed in water (Fig. 3).

The transmitter/receiver array consisted of 500 elements (element and pitch sizes: $2 \mu\text{m}$). The array was placed 2 mm above the cartilage surface and the elements were excited simultaneously with a broadband pulse (center frequency: 45 MHz, -6-dB bandwidth: 80%) to generate a plane wave. Material properties were assumed to be homogeneous, linear elastic, and isotropic and are summarized in Table I. The cartilage was divided into two zones, i.e., an upper superficial zone ($\sim 150\text{--}250 \mu\text{m}$ thick, depending on the number of layers) and a deep zone ($\sim 1750 \mu\text{m}$ thick). Cells were modeled as ellipsoids (major axis: $14 \mu\text{m}$, parallel to the cartilage surface; minor axis: $10 \mu\text{m}$) and as circles (diameter: $14 \mu\text{m}$) in the superficial and deep zones, respectively. In the superficial zone, cells were randomly placed along equidistant layers parallel to the cartilage surface (Fig. 3). Six different layer-spacing (S_c) models were created with spacing values in the range between $15 \mu\text{m}$ and $40 \mu\text{m}$ with increments of $5 \mu\text{m}$. Cells were allowed to randomly alter their depth position by

up to $\pm 2 \mu\text{m}$ relative to the layer depth. In the deep zone, cells were arranged in vertical chains consisting of 2–4 cells and the cells were allowed to randomly alter their positions in both x and depth directions by $\pm 5 \mu\text{m}$ and $\pm 7 \mu\text{m}$, respectively (Fig. 3). The spacing between adjacent chains was randomly varied between $20 \mu\text{m}$ and $30 \mu\text{m}$. The CNDs were set to $24 \times 10^3 \text{ cells/mm}^3$ and $8 \times 10^3 \text{ cells/mm}^3$ in the superficial and deep zones, respectively ([Hunziker et al., 2002](#)). In order to mimic the volumetric CND in the 2D simulations, three-dimensional (3D) CND values were converted to 2D CND values using the relation $\text{CND}_{2\text{D}} = \text{CND}_{3\text{D}}^{2/3}$. Hereinafter, only 3D CND values are reported. Random cell distribution models with identical cell sizes, shapes, and number densities as those used in organized distribution models were also used in simulation for comparison purposes.

Density (ρ) and bulk modulus (K) of cells and extracellular matrix were obtained from 250-MHz SAM experiments. A custom 250-MHz SAM system with a scanning step size of $2 \mu\text{m}$ in both lateral (x, y -plane) directions was used to image three deparaffinized cartilage sections. 2D maps of acoustical properties (i.e., acoustic impedance, speed of sound, bulk modulus, and mass density) were calculated from the digitized radio-frequency (RF) data (Fig. 2). The systems working principle, its component, and the material property estimation procedures are described in detail in [Rohrbach et al. \(2015\)](#) and [Rohrbach et al. \(2016\)](#). Only reliable estimates were included using thresholds for thickness, acoustic impedance, and speed of sound of $6\text{--}15 \mu\text{m}$, $1\text{--}2 \text{ Mrayl}$, and $1100\text{--}2000 \text{ m/s}$, respectively. The mean values and standard deviations (SDs) were calculated for cells and extracellular matrix from $0.5 \times 0.5 \text{ mm}^2$ ROI placed in the center of the amplitude map after the cells were manually segmented [Fig. 2(B)]. The segmented binary maps were eroded (i.e., disk shaped kernel with $4\text{-}\mu\text{m}$ radius) to remove artificially altered parameter estimates at tissue boundaries due to the limited resolution of the 250-MHz transducer. Shear modulus was assumed to be zero. SimSonic allows to model frequency independent attenuation following the Q-Model introduced by ([Graves, 1996](#)). Attenuation values for extracellular matrix and water

TABLE I. Material parameters for the numerical model of chondrocytes and extracellular matrix.

Material	Attenuation at 45 MHz (dB/mm)	Density (g/cm ³)	$C_{11} = C_{22}$ (GPa)	$C_{12} = C_{21}$ (GPa)	C_{44} (GPa)
Water	0.28 ^a	1.00 ^a	2.25 ^a	2.25 ^a	0.00 ^a
Chondrocytes	0.56	1.04 ^b	2.56 ^b	2.56	0.00
Extracellular matrix	10.65 ^c	1.10 ^b	2.80 ^b	2.80	0.00

^aFrom [Briggs and Kolosov \(2010\)](#).

^bDerived from SAM measurements.

^cFrom [Joiner et al. \(2000\)](#).

properties were obtained from Joiner *et al.* (2001) and Briggs and Kolosov (2010), respectively. The attenuation coefficient of chondrocytes, to our knowledge, has not been investigated and, hence, was assumed to be twice as high as the attenuation of water. The resolution of the spatial grid in x and $depth$ directions were set to $1\ \mu\text{m}$ based on the result of an initial convergence test (data not shown). The spatial simulation grid was surrounded by highly absorbing perfectly matched layers to avoid reflections from the boundaries of the simulation geometry (Bossy *et al.*, 2004).

D. Simulation model parameter variations

Based on the histological observations, three sets of artificial cartilage models with layered cell structure were designed in order to study the effects of cell structure in the superficial zone, i.e., (1) the number of aligned cell layers (N_c) in superficial zone, (2) the deviation from perfect cell alignment (V_c), and (3) the cell layer spacing (S_c). For each of the three models, the remaining constant parameters in the superficial zone were modeled as follows: number of cell layers parallel to cartilage surface $N_c = 6$; cell variation in depth within each layer $V_c = \pm 2\ \mu\text{m}$; layer spacing $S_c = 20\ \mu\text{m}$, cell major and minor axis lengths: 14 and $10\ \mu\text{m}$, respectively; $\text{CND} = 24 \times 10^3\ \text{cells/mm}^3$. The variable parameters were varied as follows: $N_c = 2, 3, 4, 5$, and 6; $V_c = 0, 2, 4, 6$, and $8\ \mu\text{m}$; $S_c = 15, 20, 25, 30, 35$, and $40\ \mu\text{m}$. For each configuration, ten realizations were created, simulated, and the results were averaged.

In addition, two sets with random cell distribution (i.e., no regularly spaced cell layers) were created to study the influence of cell size and CND on the depth-dependent frequency spectrum (DFS). Cells were randomly distributed in the superficial and deep zones. In the first set, cell dimension (i.e., major axis in the superficial and diameter in deep zones) were increased from $8\ \mu\text{m}$ to $20\ \mu\text{m}$ in steps of $2\ \mu\text{m}$. For all simulations, the aspect ratio of the cells in the superficial zone was set to 1.4 and CND was kept constant at $24 \times 10^3\ \text{cells/mm}^3$ and $8 \times 10^3\ \text{cells/mm}^3$ in the superficial and deep zones, respectively. In the second set, CNDs were increased simultaneously from 12 to 60×10^3 and from 4 to $20 \times 10^3\ \text{cells/mm}^3$ in the superficial and deep zones, respectively, while the cell size was kept constant (i.e., $14\text{-}\mu\text{m}$ major axis and $10\text{-}\mu\text{m}$ minor axis in the superficial zone, and $14\ \mu\text{m}$ in diameter in the deep zone).

E. 50-MHz QUS

Cartilage samples were interrogated using a custom ultrasound scanning device SAM200Ex, as described in a previous study (Malo *et al.*, 2013), using a spherically focused 50-MHz transducer (KSI-PT50, f -number = 3.34, diameter = 3 mm, Krämer Scientific Instruments GmbH, Herborn, Germany). The two-way -6-dB beam width in the focal plane and depth of focus, which were estimated using a wire technique (Raum and O'Brien, 1997) were $88\ \mu\text{m}$ and 2.7 mm, respectively. The center frequency measured from a pulse reflected from a planar reflector (i.e., titanium plate) in the focal plane was 42 MHz and the -6-dB frequency range was 27–55 MHz. The pulse length, defined at the -20-dB

level relative to the maximum of the signal envelope, was 64 ns. The focal distance was 9.8 mm. Samples were immersed in phosphate buffered saline ($+36\ ^\circ\text{C}$) and scanned parallel to their surface [$40\ \mu\text{m}$ step size in both x and y directions; Fig. 1(B)], with the transducer focus positioned $\sim 500\ \mu\text{m}$ below the closest surface position. This ensured that the superficial cartilage layer was within the depth of focus for the entire scan field. For each scan location, one time-resolved pulse-echo signal within a time interval of $6\ \mu\text{s}$ was stored.

F. Signal processing and spacing estimation

The following signal processing steps were applied to both experimental QUS and simulated signals to calculate AFB, AIB, and apparent cell-spacing (ACS) in the superficial cartilage layer (i.e., $<250\ \mu\text{m}$). First, the cartilage surface distance was detected using a -45-dB threshold applied to the Hilbert-transformed envelope signals (whereas 0 dB corresponds to 1 V). This distance served as reference (i.e., depth = $0\ \mu\text{m}$). Then, each QUS dataset was separated into overlapping ROI with a radius of $300\ \mu\text{m}$ in the x - y plane and a depth of $\sim 93\ \mu\text{m}$ [i.e., 117-ns time gate duration; Fig. 4(A)]. The distance between adjacent ROIs was $200\ \mu\text{m}$ (i.e., $\sim 60\%$ overlap) in the lateral direction (x - y plane) and 12.5 ns (i.e., $\sim 10.3\ \mu\text{m}$, $\sim 90\%$ overlap) in the depth direction. The time gates were positioned such that they covered a distance range from $-100\ \mu\text{m}$ to $300\ \mu\text{m}$. The simulated signals recorded from the 500 receiver elements were treated as a single ROI. In each ROI, time signals were gated using a Hamming window. For each lateral (x , y) position, a normalized DFS was calculated as follows: Within each ROI the logarithmic power spectra were averaged and normalized by subtracting a reference spectrum. The reference spectrum for QUS signals was obtained from a calibration measurement of a planar reflector (i.e., polymethylmethacrylate) positioned at the transducers focus position (Männicke *et al.*, 2016). No depth dependence for the reference spectrum was considered as the depth of focus of the transducer was large compared to the evaluation depth. In the simulations the reference signal was obtained from the cartilage surface reflection in a model without cells. A representative DFS is shown in Fig. 4(B). Within each ROI, AFB and AIB were also calculated using established procedures (Chérin *et al.*, 1998; Männicke *et al.*, 2014a).

FIG. 4. (Color online) (A) Representative B-mode image of Mankin score 3 sample (sample #3). The white surface line is the detected cartilage surface. The yellow and blue boxes correspond to one single ROI and the full evaluation depth range, respectively. (B) Representative DFS averaged from 100 RF signals taken from the center of the sample [i.e., the boxes indicated in (A)].

If multiple scatterers (cells) have similar distances in the sound propagation direction (i.e., spacing = Δtc , with $c = 1590$ m/s, Δt is the time-of-flight difference between waves scattered from adjacent scatterers), then this coherent part will add up at frequencies $f_{pk}^n = 1/\Delta t$ ($n = 1, 2, 3, \dots$), which are observed in the spectrum as a visible peak. f_{pk}^n can be converted to a cell-spacing value using relation

$$\text{spacing} = nc/2f_{pk}^n. \quad (1)$$

Based on the numerical ultrasound simulations, an algorithm was developed and optimized to detect the peak-frequency (f_{pk}) for each depth in the DFS and to estimate the cell-layer spacing. The same algorithm was then applied to the QUS-based DFS. Briefly, the DFS were analyzed in the -12 -dB bandwidth of the reference signal (i.e., 16 – 60 MHz). A frequency peak was defined as a local extrema, if the difference of its amplitude to its neighboring local minima was larger than a defined threshold. In this study, the threshold was set to 0.1 dB. If more than one peak was detected within the bandwidth, the peak closest to the center frequency was selected. Furthermore, to exclude unreliable peaks, each DFS was normalized by the maximum amplitude of the DFS and all peaks below -10 dB were excluded. The n th peak (f_{pk}^n) closest to the center frequency of the reference signal was selected. This procedure was applied to each power spectra of a single DFS yielding an ACS estimate as a function of depth for each lateral (x, y) position.

For comparison with the histology data, ACS values were averaged within regions that (i) showed a smooth surface, (ii) low surface inclination, and (iii) contained at least partially the cutting plane from which the histology sections were taken. Moreover, individual ACS values were defined to be outliers and were removed from further analysis, if they were out of the interquartile range, i.e., smaller or larger than 25th or 75th percentiles, respectively, within a particular cartilage depth. Then, similar to the histology-based analysis, ACS estimates were averaged between depths of

$100 \mu\text{m}$ and $250 \mu\text{m}$ with intervals of $25 \mu\text{m}$ yielding six average ACS values per sample. Spacing estimates between the cartilage surface and $100\text{-}\mu\text{m}$ depth were omitted because the 117 -ns gated signals were affected by the cartilage surface reflection.

G. Statistical analyses

To determine sample-specific (i.e., Mankin score) and modality-related (LM, QUS) differences of ACS estimates, Kruskal-Wallis tests (using χ^2 statistic) with *post hoc* Bonferroni correction were conducted. Pearson correlation was used to assess the relation between ACS and depth and the correlations between AIB, AFB, QUS-, and LM-based spacing. All statistical results were considered significant for $p \leq 0.05$. Statistical tests were performed using the Statistics Toolbox of MATLAB R2014a (MathWorks, Natick, MA).

III. RESULTS

A. 250-MHz SAM and LM results

Chondrocytes could be distinguished from the extracellular matrix in the 250-MHz SAM amplitude maps [Fig. 2(B)]. Mean and SDs of Z , c , ρ , and K were 1.75 ± 0.03 Mrayl, 1590 ± 20 m/s, 1.10 ± 0.01 g/cm³, and 2.80 ± 0.07 GPa for extracellular matrix, respectively, and 1.64 ± 0.01 Mrayl, 1568 ± 8 m/s, 1.04 ± 0.012 g/cm³, and 2.56 ± 0.01 GPa for chondrocytes, respectively.

The average LM-based cell spacing was $20.8 \pm 2.2 \mu\text{m}$ (mean $\pm$ SD, $N = 9$). A depth-dependent cell spacing was observed in four out of nine samples (Table II). However, the slope of the depth dependence was not related to the Mankin score.

B. Numerical ultrasound simulation results

A characteristic DFS obtained from simulation with a $20\text{-}\mu\text{m}$ spacing between layers ($N_c = 6$) in the superficial zone of cartilage is shown in Fig. 5(A). As expected, the spectrum

TABLE II. QUS- and LM-based spacing estimates, AIB and AFB (mean $\pm$ SD in the range between $100 \mu\text{m}$ and $250 \mu\text{m}$) for each sample. Slope values of linear fits for spacing vs depth are shown for significant regressions together with the R^2 values. n.s. denotes not significant.

Sample	Mankin score	QUS spacing (μm)	LM spacing (μm)	AIB (dB)	AFB (dB/MHz)	QUS spacing vs depth ($\mu\text{m}/\mu\text{m}$)	R^2	LM spacing vs depth ($\mu\text{m}/\mu\text{m}$)	R^2
Mankin score group I									
1	1	22.6 ± 3.0	20.0 ± 1.2	-28.8 ± 3.0	0.016 ± 0.05	0.07	0.50	—	n.s.
2	3	17.0 ± 3.5	17.4 ± 1.4	-25.4 ± 2.3	0.05 ± 0.06	0.06	0.50	0.02	0.48
3	3	31.8 ± 2.1^a	21.9 ± 2.8	-30.5 ± 3.8	-0.10 ± 0.05	0.04	0.71	0.02	0.35
4	3	28.1 ± 2.0^a b	24.3 ± 1.8 b	-33.1 ± 3.4 b	-0.07 ± 0.02 b	0.04	0.69	—	n.s.
Mankin score group II									
5	5	8.2 ± 0.9^a	22.1 ± 0.7	-34.4 ± 3.1	-0.05 ± 0.02	0.01	0.52	—	n.s.
6	5	32.8 ± 1.0^a	21.0 ± 3.2	-33.0 ± 2.4	-0.07 ± 0.02	0.02	0.49	—	n.s.
7	6	32.0 ± 0.5^a	21.7 ± 1.1	-31.7 ± 2.1	-0.09 ± 0.01	0.01	0.29	-0.02	0.34
8	6	31.3 ± 0.5^a	21.1 ± 5.6	-31.8 ± 1.1	-0.09 ± 0.02	0.01	0.62	0.1	0.68
9	6	30.8 ± 0.7^a b	17.4 ± 1.5 b	-33.1 ± 3.9 b	-0.11 ± 0.02 b	0.01 b	0.66	— n.s.	n.s.

^aIndicates significant differences between QUS- and LM-based spacing estimates.

^bIndicates significant differences between the Mankin score groups.

FIG. 5. Coherent backscatter analysis in structured cartilage backscatter models. (A) Depth-dependent frequency spectra (DFS) for cartilage model with regular spacing of $S_c = 20 \mu\text{m}$ in superficial zone. Zero on the depth-axis indicates the first gate position. To illustrate the impacts of number of cell lines N_c and cell variation in depth V_c , the spectra at the dashed black line position are shown in (B) and (C). (B) DFS evaluated for various number of horizontally aligned cell layers N_c : from two up to six. Random depth wise cell variation V_c is $\pm 2 \mu\text{m}$. (C) The random depth wise cell variation V_c ranges from no variation ($0 \mu\text{m}$) up to $\pm 8 \mu\text{m}$. The number of aligned cell layers N_c is six. For both cases, the cell major axis is $14 \mu\text{m}$ in x -direction and minor axis is $10 \mu\text{m}$ in the depth direction, and the CND in the superficial zone is $24 \times 10^3 \text{ cells/mm}^3$.

FIG. 6. DFS for (A) randomly distributed cells, (B)–(D) periodic structures with a spacing of (B) $20 \mu\text{m}$, (C) $30 \mu\text{m}$, and (D) $40 \mu\text{m}$. Zero on the depth-axis indicates the first gate position. (A) For the random model, a broad peak centered at 45 MHz can be seen. (B) One sharp high-intensity backscatter band between 35 MHz and 45 MHz appears in case of regular cell spacing of $20 \mu\text{m}$. For larger regular spacing (C) and (D), several narrow backscatter bands occur in the observed frequency range. For comparison, the subsurface spectra (indicated by the dashed black line) are shown in (E). The other cell distribution parameters in the superficial zone are: CND = $24 \times 10^3 \text{ cells/mm}^3$, cell major and minor axis lengths: $14 \mu\text{m}$ and $10 \mu\text{m}$; for the deep zone, number of aligned cell layers $N_c = 6$, deviation from perfect alignment $V_c = \pm 2 \mu\text{m}$.

obtained from this zone shows a local peak at around 40 MHz, which corresponds, for $n = 1$ [Eq. (1)], to the set spacing. A detectable peak was observed in the DFS, if three or more aligned cell layers (i.e., $N_c \geq 3$) were present [Fig. 5(B)]. With increasing N_c , the peak became more pronounced in amplitude and narrower in width when all other parameters were kept constant. No further improvement was observed for more than five cell layers because the gate length limited the number of cell layers contributing to the analysis. An increase in the randomness in the depth position of cells in a single layer (V_c) led to a reduction of peak amplitude and an increase in peak width [Fig. 5(C)]. When the deviation from a perfect cell alignment reached $V_c = 8 \mu\text{m}$, the peak completely diminished.

The variation of the cell layer spacing S_c showed a characteristic shift of the coherent peak positions in the DFS. For $S_c = 20 \mu\text{m}$, one peak was observed at $\sim 39 \text{ MHz}$ [Fig. 6(B)]. An increase of S_c to $30 \mu\text{m}$ [Fig. 6(C)] shifted that peak toward a lower frequency ($\sim 25 \text{ MHz}$) and a second peak occurred at $\sim 54 \text{ MHz}$. A further increase of S_c to $40 \mu\text{m}$ [Fig. 6(D)] yielded three peaks in the spectrum at frequencies of $\sim 16 \text{ MHz}$, $\sim 39 \text{ MHz}$, and $\sim 60 \text{ MHz}$ [Fig. 6(E)]. In contrast to the structured models, the random model [Fig. 6(A)] exhibited the characteristic broad peak caused by incoherent backscatter, which was affected by cell-size and cell-number density (Fig. 7).

While an increasing cell size changes the frequency dependent backscatter intensity, an increase of the CND resulted in a non-linear and rather frequency-independent increase of the backscatter amplitude. Note that (i) all peaks obtained from random models were much wider than those obtained from structured models, and (ii) the spectrum obtained from the model with $12\text{-}\mu\text{m}$ cell major axis [Fig. 7(B)] is very similar to the one obtained with a two-layer structure, indicating that more than two layers are required to produce a coherent backscatter amplitude that is larger than that of the incoherent backscatter.

A strong correlation was observed between the spacing defined in the model and the values (i.e., ACS values) determined by the spacing algorithm [Fig. 5(F)]. The spacing algorithm failed (i.e., no peak was detected) in 1% of all cases when applied to models with layered structure, while it failed in 97% of cases when applied to the randomized models.

In the random models, an increase of CND resulted in the expected non-linear increase of AIB ($R^2 = 0.94$), but did

not affect AFB. A change of the cell size (i.e., major axis) had a minor effect on AIB for cells larger than $8 \mu\text{m}$, while AFB continuously decreased from 0.15 dB/MHz to -0.25 dB/MHz for cell sizes of $8 \mu\text{m}$ – $16 \mu\text{m}$, respectively, and increased again to -0.15 dB/MHz for the largest simulated cell size of $20 \mu\text{m}$ (data not shown). For the structured models, no consistent dependencies of AIB and AFB with the simulated cell morphology could be observed. It should be noted, however, that due to the sharp coherent peaks in the spectra, the linear fit used for the calculation of AFB is not feasible, and AIB strongly depends on the peak position relative the integration boundaries.

C. QUS-based cell spacing

ACS estimates were significantly lower ($p < 0.001$) in the group with small Mankin scores (≤ 3) ($24.9 \pm 6.5 \mu\text{m}$, mean $\pm$ SD), when compared to the group with high-Mankin scores (≥ 5 ; $31.0 \pm 1.7 \mu\text{m}$; Table II). A similar trend was not observed in the spacing estimated from LM. No significant inter-specimen differences were found for both QUS- and LM-based spacing estimates. All nine samples showed a moderate to strong positive correlation with cartilage-depth with an average R^2 of 0.55 and slope of $3 \mu\text{m}$ per $100 \mu\text{m}$ cartilage depth (Table II).

A comparison between QUS- and LM-based spacing revealed that QUS-based ACS was significantly higher compared to LM-based spacing ($p < 0.01$). However, this effect was stronger for samples with high-Mankin scores ($p < 0.001$, $\chi^2 = 42.9$) compared to the effect found in the lower Mankin score group ($p < 0.05$, $\chi^2 = 6.1$). A strong correlation ($R^2 = 0.59$, $p < 0.001$) between QUS- and LM-based spacing estimates was found in the low-Mankin score group (Fig. 8), but not for the high-Mankin score group. After pooling all data, the correlation was weak ($R^2 = 0.13$, $p < 0.05$).

AFB and AIB were significantly ($p < 0.05$) different between the low (AIB, $-29.5 \pm 3.9 \text{ dB}$; AFB, $-0.02 \pm 0.08 \text{ dB/MHz}$) and high (AIB, $-32.8 \pm 2.4 \text{ dB}$; AFB, $-0.09 \pm 0.02 \text{ dB/MHz}$) Mankin-score groups (see also Table II). Moreover, AFB was highly and moderately correlated with ACS estimates from QUS [$R^2 = 0.89$, $p < 0.0001$; Fig. 8(B)] and LM [$R^2 = 0.64$, $p < 0.0001$, data not shown], respectively. Moreover, a moderate correlation was observed between QUS-based ACS and AIB ($R^2 = 0.53$, $p < 0.0001$, data not shown).

FIG. 7. DFS for cartilage models with random cell spacing. (A) Cell size: $14 \mu\text{m} \times 10 \mu\text{m}$, CND of $24 \times 10^3 \text{ cells/mm}^3$ in the superficial zone. Zero on the depth-axis indicates the first gate position. To illustrate the impacts of cell size and density, the subsurface spectra (indicated by the dashed black line) are shown in (B) and (C). (B) At a cell density of $24 \times 10^3 \text{ cells/mm}^3$, the variation of cell size results in changes of the frequency-dependent backscatter intensity. (C) An increase of cell density at fixed cell size ($14 \mu\text{m} \times 10 \mu\text{m}$) results in a rather frequency-independent amplitude increase.

FIG. 8. (A) Linear correlation between LM and QUS-based spacing estimates from samples of the low-Mankin group ($N=4$). From each individual depth-dependent cell-spacing profile, the values in the range between $100\ \mu\text{m}$ and $250\ \mu\text{m}$ were averaged in intervals of $25\ \mu\text{m}$, yielding six spacing values per sample. (B) Linear correlation between AFB and QUS-based spacing estimates including all samples.

IV. DISCUSSION

This study provides evidence that a regular layered organization of chondrocytes in human hyaline cartilage is a potential cause of coherent ultrasound scattering. We applied a simple peak detection algorithm that allows estimating cell spacing based on the resonance peak frequency. Numerical simulations confirmed that a regular layered organization would lead to resonance peaks in the frequency spectrum of the backscattered signals.

In the simulations, the developed peak detection algorithm was able to detect the cell spacing with an average absolute error of $0.2\ \mu\text{m}$. When the algorithm was applied to the experimental data, a significant correlation between LM- and QUS-based spacing estimates was found, providing evidence that the resonance peak detected in the frequency range between 23 and 56 MHz is indeed associated with the spacing of chondrocyte layers in intact human hyaline cartilage. However, in samples that exhibited considerable surface degradation (Mankin score ≥ 5), the correlation between QUS and LM-spacing diminished.

The conventional analysis of ultrasound backscatter from AC uses AIB and AFB (Chérin *et al.*, 1998; Männicke *et al.*, 2014a). In the current study, both AIB and AFB were found to decrease with increasing Mankin scores. This is in line with findings in which both parameters were found to be related to changes in the organization of the extracellular matrix, collagen fiber orientation, CND, and specific cell alignment and were proposed as potentially good indicators for signs of early OA (Inkinen *et al.*, 2015; Männicke *et al.*, 2014a; Männicke *et al.*, 2016). We found that both AIB and AFB were correlated with the QUS- and LM-based spacing estimates in samples with low-Mankin scores (≤ 3). However, if the backscatter spectrum is composed of both coherent and incoherent backscatter contributions, the latter has a considerable impact on the parameter estimates. AFB assesses the frequency dependence (assuming a smooth linear change within the evaluation bandwidth) and AIB the magnitude of the power spectrum. It is well known that for incoherent backscatter, the frequency dependence is related to shape, size, and elastic properties of the scattering structures, whereas the magnitude is related to the size, number density, and impedance contrast of the scatterers (Insana *et al.*, 1990). In a sparse medium with randomly distributed scatterers, the

characteristics of the averaged normalized power spectrum depend only on incoherent scattering, which can be described by the backscatter coefficient and attenuation. The random scatterer positions act as noise and the expected contribution to the backscatter coefficient is zero. However, if a sufficiently large number of scatterers have a similar distance between each other (spacing = $\Delta t c_0$, with c_0 = speed of sound in the background medium) in direction of the incident wave, the coherent part will add up at a frequency of $1/\Delta t$ and can be observed in the normalized power spectrum as a peak. However, other reasons for a non-linear frequency dependency of the normalized power spectrum amplitude can be considered, e.g., multiple scattering or other structure effects due to a denser scattering medium. These effects were not investigated in this study but should be taken into account in future studies.

Calculating AIB and AFB without the consideration of the potential contribution of coherent backscatter leads to parameter estimates, which are strongly dependent on the selected bandwidth of the transducer and the evaluated frequency range if coherent backscatter occurs. Therefore, future research should focus on the detection and separation of coherent from incoherent backscatter contributions, yielding system-independent QUS parameters related to cell morphology. This could be achieved by employing (i) a larger bandwidth and (ii) more sophisticated signal processing approaches, such as cepstral analysis and other well established methods (Wear *et al.*, 1993).

Numerical simulations showed that changes in regularity of the layered cell distribution would lead to a broadening, weakening, or even disappearing of the frequency peak. For example, early OA changes in the extracellular matrix (e.g., collagen network degradation) of the superficial zone cause swelling of the tissue, which may further affect cell spacing as well as the acoustic properties of extracellular matrix. Hypercellularity (i.e., increase in cell number), which also may occur in the early OA, will increase the frequency independent intensity of the backscatter amplitude. Moreover, when OA further progresses, the superficial zone starts to wear off exposing the transitional and deep zones, in which the cell size is bigger and cell distribution and spacing are different. These OA-related changes in the superficial zone may explain the observed increase in the QUS-based ACS estimates in more degenerated cartilage samples

($p < 0.001$, Mankin ≤ 3 vs Mankin 5 and 6). Therefore, together with other QUS estimates the proposed cell spacing estimation can serve as an additional biomarker for diagnosing early signs of OA and improve the overall performance and reliability of future ultrasound-based OA diagnostic tools. These tools can be clinically applied using minimally invasive approaches such as ultrasound arthroscopy, which is a promising method to assess cartilage integrity (Kaleva *et al.*, 2011; Liukkonen *et al.*, 2014; Puhakka *et al.*, 2016; Spahn *et al.*, 2011). Non-invasive QUS approaches could also be considered because several studies suggest that a significant portion of weight bearing surfaces in the knee can be assessed transcutaneously using clinical available scanners (Grassi *et al.*, 1999; Möller *et al.*, 2008; Naredo *et al.*, 2009). Nevertheless, studies need to investigate whether the bandwidth used in this study can be applied *in vivo* in humans or whether QUS using lower frequencies is still sensitive to OA related changes in properties of chondrocytes and cartilage matrix. We selected the bandwidth in this work based on our previous studies that indicated the appearance of coherent backscatter (Männicke *et al.*, 2016). Nevertheless, our data also suggest that a bandwidth with a lower center frequency could still detect relevant spacing. For example, a 25-MHz transducer with a 60% -6 -dB bandwidth would still permit detecting spacing as small as $23\text{ }\mu\text{m}$.

The potential of QUS being sensitive to properties of chondrocytes is of particular interest since cell number, metabolic activity, and apoptosis are known to play a crucial role in the early development of OA (Hwang and Kim, 2015). Based on the results of this study, we hypothesize that variations in cell spacing will have a significant impact on ultrasound scattering properties and can be quantified using novel QUS approaches. Future studies will investigate whether backscatter models of human cartilage are able to describe coherent and incoherent scattering separately. Therefore, QUS has the potential to become a leading and innovative diagnostic and monitoring tool for OA.

The limitations of this study are as follows: The *in silico* cartilage models developed in our study were based on material properties of extracellular matrix and chondrocytes derived from high-resolution 250-MHz SAM. These measurements provided the most realistic material parameters for the models currently possible and these values have not been previously reported for chondrocytes in literature. However, material properties estimated from 250-MHz SAM need to be considered with caution. Embedding, de-paraffinization and rehydration of the samples may have altered the tissue properties (Hunziker *et al.*, 2014). Further studies are required to assess more natural material properties, for example, from cryo-sectioned samples. However, speed of sound and density values for extracellular cartilage matrix reported in the present study are in good agreement with values reported in a previous study of Myers *et al.* (1995). In contrast, the study by Leicht and Raum (2008) reported higher acoustic impedance values for extracellular cartilage matrix (i.e., 2.12 ± 0.02 Mrayl) measured with a 50-MHz acoustic microscope. However, in that study the tissue was prepared and measured in hyperosmolar saline solution, which may explain the higher values compared to the values

obtained in the present study. Tepic *et al.* (1983) reported cartilage-to-saline acoustic impedance ratios of 1.1–1.2, which is consistent with our findings ($1.8\text{ Mrayl}/1.5\text{ Mrayl} = 1.2$). Another limitation of our SAM data is the impossibility to reliably map attenuation coefficients obtained at 250 MHz to much lower frequencies, since the frequency dependence of attenuation cannot be assumed to be linear over such a large frequency range (Chivers and Hill, 1975; Parker *et al.*, 1984). Therefore, attenuation values used in simulations were obtained from surveying the literature (Briggs and Kolosov, 2010; Joiner *et al.*, 2000).

Even though correlation was observed between the LM- and QUS-based cell spacing estimates, a bias between the two estimates was observed. Both algorithms for estimating cell spacing by means of QUS and LM were optimized for cases in which a spacing is apparent. Furthermore, the spacing estimation from the LM sections was performed on $5\text{-}\mu\text{m}$ thick 2D sections, while the QUS-based spacing was calculated from volumetric data in 3D, which is another potential reason for the observed differences. In future studies, this could be taken into account by estimating the cell spacing from volumetric data, e.g., from confocal microscopy. Nevertheless, the moderate correlation between QUS- and LM-based spacing estimates in the low-Mankin score group suggests that in hyaline cartilage with a non-destroyed collagen architecture in the superficial zone, the frequency of the detected resonance peak in the superficial zones may be linked to the spacing between cell layers.

Another limitation of this study is the limited sample size of two donors with signs of mild to severe OA. We are planning to conduct *ex vivo* experiments with larger sample sizes and wider ranges of degenerated OA cartilage to confirm the findings of this study. Nevertheless, the samples used in this study exhibited a wide and representative range of early degeneration and our results motivate further research.

Furthermore, our simulations considered only variations in the cellular structure. Effects originating from variations of extracellular matrix properties were not considered. However, it has been shown that the collagen network of the extracellular matrix may affect the backscattered ultrasound spectrum (Inkinen *et al.*, 2014; Männicke *et al.*, 2016). Finally, it should be noted that the implemented spacing estimation method is based on a simple peak detection. This approach will only work sufficiently, if the spacing between chondrocyte layers matches the frequency range of the transducer. In the current analysis, the lower bandwidth limit was 16 MHz, which is equivalent to a spacing of $\sim 50\text{ }\mu\text{m}$. For larger spacing, higher harmonics will appear in the bandwidth instead of the fundamental frequency leading to falsely detected lower spacing values. This can be significantly improved, if two or more peaks can be detected within the bandwidth allowing estimating the fundamental frequency. A similar approach has been implemented in our SAM analysis procedures (Rohrbach *et al.*, 2016). Nevertheless, the current estimation method is sufficient for spacing values in the range between 13 and $50\text{ }\mu\text{m}$ and larger spacing values are not expected in the superficial zone of hyaline cartilage in the early stages of OA.

Future studies should also investigate the impact of anatomical variation in cell organization on ultrasound backscatter measurements. The study by Rolaufts *et al.* (2008) showed significant variation of chondrocyte organization between tibia, femur, and patella. The authors found that chondrocytes form clusters of different shapes, which will have an effect on the backscattered ultrasound signals. We expect that anatomical-site-specific ultrasound-backscattering and OA-classification models will further improve future QUS applications.

V. CONCLUSION

The results of the present study suggest that regular spacing of cell layers in the superficial zone of intact AC can be estimated using QUS. If cartilage shows signs of degradation, our preliminary results indicate that the estimated spacing is increased. This is expected since, in particular, early OA-related changes such as slight surface degradation, cartilage swelling, and changes of the extracellular matrix properties will directly affect the spacing between the cell layers. It is expected that these morphological changes will have a direct impact on the backscattered frequency spectrum. The potential of coherent scattering of chondrocytes does not only have important implications for the correct estimation and interpretation of established QUS parameters, such as AFB and AIB, but could also improve the assessment of cartilage integrity during the early stages of OA. A separated analysis of coherent and incoherent scatter may lead to QUS-based biomarkers that can detect early signs of OA. Our results emphasize the strong potential of QUS as a non-invasive, fast, and inexpensive diagnostic and monitoring tool for early-stage OA-related cartilage pathogenesis. QUS could be used as inexpensive primary health care level monitoring option alongside the current state-of-the-art modalities, such as MRI for assessing the effectiveness of novel OA-treatment approaches, such as disease-modifying OA drugs, autologous chondrocyte implantation, or microfracturing (Brittberg and Winalski, 2003). However, further experimental *ex vivo* and *in vivo* studies are needed with larger sample sizes and wider ranges of degenerated OA cartilage, as well as more detailed numerical models are needed to fully investigate the potential of QUS for monitoring and diagnosing OA.

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¹www.simsonic.fr (Last viewed April 19, 2017).

- Aula, A. S., Töyräs, J., Tiitu, V., and Jurvelin, J. S. (2010). “Simultaneous ultrasound measurement of articular cartilage and subchondral bone,” *Osteoarthritis Cartilage* **18**, 1570–1576.
- Bossy, E., Talmant, M., and Laugier, P. (2004). “Three-dimensional simulations of ultrasonic axial transmission velocity measurement on cortical bone models,” *J. Acoust. Soc. Am.* **115**, 2314–2324.
- Briggs, A., and Kolosov, O. V. (2010). *Acoustic Microscopy (Monographs on the Physics and Chemistry of Materials)*, 2nd ed. (Oxford University Press, Oxford), pp. 1–356.
- Brittberg, M., and Winalski, C. S. (2003). “Evaluation of cartilage injuries and repair,” *J. Bone Joint Surg. Am.* **85**, 58–69.
- Buckwalter, J. A., and Mankin, H. J. (1998). “Articular cartilage: Tissue design and chondrocyte-matrix interactions,” *Instr. Course Lect.* **47**, 477–486.
- Buckwalter, J. A., Mankin, H. J., and Grodzinsky, A. J. (2005). “Articular cartilage and osteoarthritis,” *Instr. Course Lect.* **54**, 465–480.
- Chérin, E., Saied, A., Laugier, P., Netter, P., and Berger, G. (1998). “Evaluation of acoustical parameter sensitivity to age-related and osteoarthritic changes in articular cartilage using 50-MHz ultrasound,” *Ultrasound Med. Biol.* **24**, 341–354.
- Chivers, R. C., and Hill, C. R. (1975). “Ultrasonic attenuation in human tissue,” *Ultrasound Med. Biol.* **2**, 25–29.
- Couture, O., Chérin, E., and Foster, F. S. (2007). “Model for the ultrasound reflection from micro-beads and cells distributed in layers on a uniform surface,” *Phys. Med. Biol.* **52**, 4189–4204.
- Franceschini, E., Guillermin, R., Tourniaire, F., Roffino, S., Lamy, E., and Landrier, J.-F. (2014). “Structure factor model for understanding the measured backscatter coefficients from concentrated cell pellet biophantoms,” *J. Acoust. Soc. Am.* **135**, 3620–3631.
- Gold, G. E., Chen, C. A., Koo, S., Hargreaves, B. A., and Bangerter, N. K. (2009). “Recent advances in MRI of articular cartilage,” *AJR Am. J. Roentgenol.* **193**, 628–638.
- Grassi, W., Lamanna, G., Farina, A., and Cervini, C. (1999). “Sonographic imaging of normal and osteoarthritic cartilage,” *Semin. Arthritis Rheum.* **28**, 398–403.
- Graves, R. W. (1996). “Simulating seismic wave propagation in 3D elastic media using staggered-grid finite differences,” *Bull. Seismol. Soc. Am.* **86**, 1091–1106.
- Hunziker, E. B., Lippuner, K., and Shintani, N. (2014). “How best to preserve and reveal the structural intricacies of cartilaginous tissue,” *Matrix Biol. J. Int. Soc. Matrix Biol.* **39**, 33–43.
- Hunziker, E. B., Quinn, T. M., and Häuselmann, H.-J. (2002). “Quantitative structural organization of normal adult human articular cartilage,” *Osteoarthritis Cartilage* **10**, 564–572.
- Hwang, H., and Kim, H. (2015). “Chondrocyte apoptosis in the pathogenesis of osteoarthritis,” *Int. J. Mol. Sci.* **16**, 26035–26054.
- Inkinen, S. I., Liukkonen, J., Tiitu, V., Viren, T., Jurvelin, J. S., and Töyräs, J. (2015). “Ultrasound backscattering is anisotropic in bovine articular cartilage,” *Ultrasound Med. Biol.* **41**, 1958–1966.
- Inkinen, S., Liukkonen, J., Ylärinne, J. H., Puhakka, P. H., Lammi, M. J., Viren, T., Jurvelin, J. S., and Töyräs, J. (2014). “Collagen and chondrocyte concentrations control ultrasound scattering in agarose scaffolds,” *Ultrasound Med. Biol.* **40**, 2162–2171.
- Insana, M. F., Wagner, R. F., Brown, D. G., and Hall, T. J. (1990). “Describing small-scale structure in random media using pulse-echo ultrasound,” *J. Acoust. Soc. Am.* **87**, 179–192.
- Jadin, K. D., Wong, B. L., Bae, W. C., Li, K. W., Williamson, A. K., Schumacher, B. L., Price, J. H., and Sah, R. L. (2005). “Depth-varying density and organization of chondrocytes in immature and mature bovine articular cartilage assessed by 3d imaging and analysis,” *J. Histochem. Cytochem. Off. J. Histochem. Soc.* **53**, 1109–1119.
- Joiner, G. A., Bogoch, E. R., Pritzker, K. P., Buschmann, M. D., Chevrier, A., and Foster, F. S. (2001). “High frequency acoustic parameters of

- human and bovine articular cartilage following experimentally-induced matrix degradation," *Ultrason. Imaging* **23**, 106–116.
- Joiner, G. A., Bogoch, E. R., Pritzker, K. P., Buschmann, M. D., and Foster, F. S. (2000). "Changes in acoustic parameters at 30 MHz of human and bovine articular cartilage following experimentally-induced matrix degradation to simulate early osteoarthritis," in *Ultrason. Symp. 2000 IEEE*, pp. 1337–1340.
- Kaleva, E., Virén, T., Saarakkala, S., Sahlman, J., Sirola, J., Puhakka, J., Paatela, T., Kröger, H., Kiviranta, I., Jurvelin, J. S., and Töyräs, J. (2011). "Arthroscopic ultrasound assessment of articular cartilage in the human knee joint: A potential diagnostic method," *Cartilage* **2**, 246–253.
- Leicht, S., and Raum, K. (2008). "Acoustic impedance changes in cartilage and subchondral bone due to primary arthrosis," *Ultrasonics* **48**, 613–620.
- Li, X., and Majumdar, S. (2013). "Quantitative MRI of articular cartilage and its clinical applications," *J. Magn. Reson. Imaging* **38**, 991–1008.
- Liukkonen, J., Lehenkari, P., Hirvasniemi, J., Joukainen, A., Viren, T., Saarakkala, S., Nieminen, M. T., Jurvelin, J. S., and Töyräs, J. (2014). "Ultrasound arthroscopy of human knee cartilage and subchondral bone *in vivo*," *Ultrason. Med. Biol.* **40**, 2039–2047.
- Malo, M. K. H., Rohrbach, D., Isaksson, H., Töyräs, J., Jurvelin, J. S., Tamminen, I. S., Kröger, H., and Raum, K. (2013). "Longitudinal elastic properties and porosity of cortical bone tissue vary with age in human proximal femur," *Bone* **53**, 451–458.
- Mankin, H. J., and Lippello, L. (1970). "Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips," *J. Bone Jt. Surg. Am.* **52**, 424–434.
- Männicke, N., Schöne, M., Gottwald, M., Göbel, F., Oelze, M. L., and Raum, K. (2014a). "3-D high-frequency ultrasound backscatter analysis of human articular cartilage," *Ultrason. Med. Biol.* **40**, 244–257.
- Männicke, N., Schöne, M., Liukkonen, J., Facht, D., Inkinen, S., Malo, M. K., Oelze, M. L., Töyräs, J., Jurvelin, J. S., and Raum, K. (2016). "Species-independent modeling of high-frequency ultrasound backscatter in hyaline cartilage," *Ultrason. Med. Biol.* **42**, 1375–1384.
- Männicke, N., Schöne, M., Oelze, M., and Raum, K. (2014b). "Articular cartilage degeneration classification by means of high-frequency ultrasound," *Osteoarthritis Cartilage* **22**, 1577–1582.
- Möller, I., Bong, D., Naredo, E., Filippucci, E., Carrasco, I., Moragues, C., and Iagnocco, A. (2008). "Ultrasound in the study and monitoring of osteoarthritis," *Osteoarthritis Cartilage* **16**, S4–S7.
- Mow, V. C., Ratcliffe, A., and Poole, A. R. (1992). "Cartilage and diarthrodial joints as paradigms for hierarchical materials and structures," *Biomaterials* **13**, 67–97.
- Myers, S. L., Dines, K., Brandt, D. A., Brandt, K. D., and Albrecht, M. E. (1995). "Experimental assessment by high frequency ultrasound of articular cartilage thickness and osteoarthritic changes," *J. Rheumatol.* **22**, 109–116.
- Naredo, E., Acebes, C., Möller, I., Canillas, F., de Agustín, J. J., de Miguel, E., Filippucci, E., Iagnocco, A., Moragues, C., Tuneu, R., Uson, J., Garrido, J., Delgado-Baeza, E., and Sáenz-Navarro, I. (2009). "Ultrasound validity in the measurement of knee cartilage thickness," *Ann. Rheum. Dis.* **68**, 1322–1327.
- Nieminen, H. J., Zheng, Y., Saarakkala, S., Wang, Q., Töyräs, J., Huang, Y., and Jurvelin, J. (2009). "Quantitative assessment of articular cartilage using high-frequency ultrasound: Research findings and diagnostic prospects," *Crit. Rev. Biomed. Eng.* **37**, 461–494.
- Parker, K. J., Lerner, R. M., and Waag, R. C. (1984). "Attenuation of ultrasound: Magnitude and frequency dependence for tissue characterization," *Radiology* **153**, 785–788.
- Puhakka, J., Afara, I. O., Paatela, T., Sormaala, M. J., Timonen, M. A., Virén, T., Jurvelin, J. S., Töyräs, J., and Kiviranta, I. (2016). "In vivo evaluation of the potential of high-frequency ultrasound for arthroscopic examination of the shoulder joint," *Cartilage* **7**, 248–255.
- Raum, K., and O'Brien, W. D. (1997). "Pulse-echo field distribution measurement technique for high-frequency ultrasound sources," *IEEE Trans. Ultrason. Ferroelectr. Freq. Control* **44**, 810–815.
- Rohrbach, D., Jakob, A., Lloyd, H., Tretbar, S., Silverman, R. H., and Mamou, J. (2016). "A novel quantitative 500-MHz acoustic-microscopy system for ophthalmologic tissues," *IEEE Trans. Biomed. Eng.* **64**, 715–724.
- Rohrbach, D., Lloyd, H. O., Silverman, R. H., and Mamou, J. (2015). "Fine-resolution maps of acoustic properties at 250 MHz of unstained fixed murine retinal layers," *J. Acoust. Soc. Am.* **137**, EL381–EL387.
- Rolaufts, B., Williams, J. M., Grodzinsky, A. J., Kuettner, K. E., and Cole, A. A. (2008). "Distinct horizontal patterns in the spatial organization of superficial zone chondrocytes of human joints," *J. Struct. Biol.* **162**, 335–344.
- Schöne, M., Männicke, N., Gottwald, M., Göbel, F., and Raum, K. (2013). "3-D high-frequency ultrasound improves the estimation of surface properties in degenerated cartilage," *Ultrason. Med. Biol.* **39**, 834–844.
- Spahn, G., Klinger, H. M., Baums, M., Pinkepank, U., and Hofmann, G. O. (2011). "Reliability in arthroscopic grading of cartilage lesions: Results of a prospective blinded study for evaluation of inter-observer reliability," *Arch. Orthop. Trauma Surg.* **131**, 377–381.
- Tepic, S., Macirowski, T., and Mann, R. W. (1983). "Mechanical properties of articular cartilage elucidated by osmotic loading and ultrasound," *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3331–3333.
- Thomas, C. M., Fuller, C. J., Whittles, C. E., and Sharif, M. (2007). "Chondrocyte death by apoptosis is associated with cartilage matrix degradation," *Osteoarthritis Cartilage* **15**, 27–34.
- Virén, T., Saarakkala, S., Jurvelin, J. S., Pulkkinen, H., Tiitu, V., Valonen, P., Kiviranta, I., Lammi, M., J., and Töyräs, J. (2010). "Quantitative evaluation of spontaneously and surgically repaired rabbit articular cartilage using intra-articular ultrasound method *in situ*," *Ultrason. Med. Biol.* **36**, 833–839.
- Wear, K. A., Wagner, R. F., Insana, M. F., and Hall, T. J. (1993). "Application of autoregressive spectral analysis to cepstral estimation of mean scatterer spacing," *IEEE Trans. Ultrason. Ferroelectr. Freq. Control* **40**, 50–58.