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Ex-vivo quantitative ultrasound assessment of cartilage degeneration

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***Abstract*—Osteoarthritis is a common disease that implies joint degeneration and that strongly affects the quality of life. Conventional radiography remains currently the most used diagnostic method, even if it allows only an indirect assessment of the articular cartilage and employ the use of ionizing radiations. A non-invasive, continuous and reliable diagnosis is crucial to detect impairments and to improve the treatment outcomes.**

Quantitative ultrasound techniques have proved to be very useful in providing an objective diagnosis of several soft tissues. In this study, we propose quantitative ultrasound parameters, based on the analysis of radiofrequency data derived from both healthy and osteoarthritis-mimicking (through chemical degradation) *ex-vivo* cartilage samples. Using a transmission frequency typically employed in the clinical practice (7.5-15 MHz) with an external ultrasound probe, we found results in terms of reflection at the cartilage surface and sample thickness comparable to those reported in the literature by exploiting arthroscopic transducers at high frequency (from 20 to 55 MHz). Moreover, for the first time, we introduce an objective metric based on the phase entropy calculation, able to discriminate the healthy cartilage from the degenerated one.

***Clinical Relevance*— This preliminary study proposes a novel and quantitative method to discriminate healthy from degenerated cartilage. The obtained results pave the way to the use of quantitative ultrasound in the diagnosis and monitoring of knee osteoarthritis.**

I. Introduction

Osteoarthritis (OA) is a common degenerative disease that involves mainly synovial joints and has a higher prevalence among older people [1]. Pain and functional joint disability caused by OA negatively affect the quality of life of the patients affected by this pathology. Currently, conventional radiography is the most widely used diagnosis technique, however it does not allow a precise and accurate assessment of the cartilage especially in the early stage of OA and besides it is considered hazardous due to the use of ionizing radiations [2]. Hence, an objective, non-invasive, and thereby repeatable diagnosis of OA is essential to prevent any complications and thus to improve the treatment outcomes.

Among current imaging methods, ultrasound (US) has proved to be a useful diagnostic tool for knee disorders [3]. However, its adoption in the clinical practice is hampered by some intrinsic limitations. Indeed, conventional B-mode imaging examinations give morphological information which are basically qualitative and subjective being both system- and operator-dependent.

Quantitative ultrasound (QUS) techniques can be used to extract objective metrics related to tissue microstructure, by working directly on the radiofrequency (RF) data derived from the piezoelectric elements of the US probe. Over the last decade, QUS has been explored to improve the diagnoses of several soft tissues, *e.g.,* for the classification of tumors, for the detection of liver diseases, for monitoring therapies, etc. [4]. The use of QUS for evaluating the degree of cartilage degeneration has also been investigated, trying to correlate the acquired backscattered US signals with alterations in the cartilage content and architecture [5].

Saarakkala *et al*. [6], explored high-frequency QUS (20 MHz) to analyze *ex-vivo* bovine articular cartilage after mechanical and enzymatic degradation. The authors found that the enzymatic treatment induced variations in the acoustic response of the cartilage, such as the reflection index (RI) at the cartilage surface, the ultrasound roughness index (URI) and the spatial variation of US reflection. In another study, the same authors found a significant increase of URI in bovine cartilage with a local degenerated surface [7]. Wang *et al*. [8] also used high-frequency US (40 MHz central frequency) to analyze the surface integrity, the thickness and the acoustic properties of normal and enzymatically degraded articular cartilage. Interesting results were also found in OA-induced animal models: Niu *et al*. [9] observed a good correlation of acoustic parameters (such as URI and RI) with the OA grade in rabbit knees, using high-frequency US (55 MHz central frequency).

However, all the above-mentioned studies employed transducers working at high frequencies (≥ 20 MHz). Due to the low *in-vivo* penetration capability, US at that frequency can be clinically used only if integrated into arthroscopic tools. Moreover, most of the calculated parameters need a normalization step with respect to a reference signal, to make the measurement reliable and to remove possible artifacts and dependency from the acquisition system. In the clinical setting, such normalization step results in a time-consuming procedure, with a high probability of errors due to the employment of reference phantoms with acoustic properties not really reflecting the ones of the tissues of interest [10].

Recently, we proposed a novel reference-free parameter based on a combination of phase entropy (sampEn) and

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amplitude information. Such a parameter allowed discriminating different concentrations of calcium carbonate and hydroxyapatite (mimicking bone at different mineralization phases) by exploiting an external clinical probe with a frequency range normally used in diagnostic sonography (7.5-15MHz) [11].

In this study, the same US probe and the same reference-free approach was used to quantitatively measure US-derived parameters correlated with cartilage degradation.

II. Materials and Methods

A. Sample preparation and experimental setup

Three mature bovine patellae without noticeable lesions were purchased from a local market and stored at 4 °C until carrying out further steps. Each patella was cut using a bone biopsy instrument. Overall, 12 cylindrical cartilage samples with a diameter of 8 mm were obtained. The specimens were placed and fixed in an agarose support provided with ad-hoc holes as shown in Figure 1a. The agarose supports were prepared by dissolving low-melt agarose powder (9414, Sigma Aldrich) in deionized water at a concentration of 2% w/v and cooled at room temperature directly in the sample holder. Such a concentration of agarose shows acoustic properties and echogenicity similar to the water ones [12].

The degeneration of the articular cartilage was achieved by exploiting the chemical action of two enzymes: trypsin and collagenase. Trypsin produces proteoglycan digestion with a slight effect on the collagen network, while collagenase is responsible for the degradation of the collagen network [8]. The healthy cartilage samples (*i.e.,* the control samples, n= 12) were first analyzed by US and then divided into two groups: trypsin treatment (n=6) and collagenase treatment (n=6). For the trypsin treatment, specimens were immersed in a 0.25% trypsin-ethylenediamtetra-acetic acid (EDTA) (59428C, Sigma Aldrich) solution at 37 °C for 4 h, as described by Wang *et al.* [8]. For the collagenase treatment, the specimens were placed in a 4 mg/mL collagenase solution (SCR103, Sigma Aldrich), at 37 °C for 24 h [8]. At the end of both treatments, all the samples were analyzed again by US.

The experimental setup used to acquire US data from the cartilage samples is depicted in Figure 1b and described in more detail in [11]. Briefly, it included a tank filled with deionized and degassed water, a support for the US probe and a sample holder. The sample holder was fixed through screws to the support in the water tank during the US scanning. US measurements were carried out using an ArtUS EXT-1H system (Telemed, Italy) equipped with a 192 elements linear probe L15-7H40-A5 (7.5-1 5MHz) The RF data were acquired from each specimen in the sample holder by using a dedicated software interface and adjusting the focus in the middle of the samples. The transmitting frequency was set at 15 MHz.

B. Morphology of cartilage samples

Scanning electron microscopy (SEM) was performed to examine the morphology of a representative sample for each experimental group (*i.e.,* control, trypsin and collagenase) to verify the effect of the chemical degradation. The samples were fixed and dehydrated using the protocol described in [13] and gold-sputtered before the analysis. SEM scans were carried out using the microscope EVO MA10 (Zeiss), setting a beam voltage of 10 kV at a working distance of around 10 mm with a resolution of 2µm.

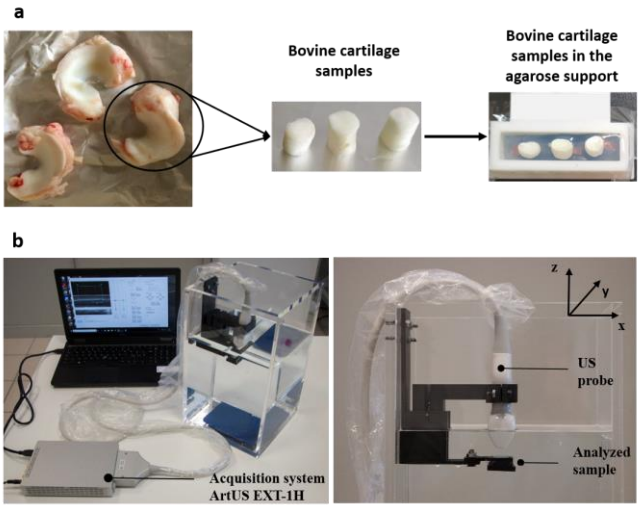

Figure 1 - Sample preparation and experimental setup. a) Preparation of ex-vivo cartilage samples: three bovine patellae were cut using a bone biopsy instrument and then placed in an agarose support within the sample holder. b) Experimental setup, including the acquisition system, the support for the probe and the sample holder used for RF signals acquisition.

C. Ultrasound data processing

All data were collected setting an acquisition window in the US software interface with the same dimensions for all the samples. Since we were not interested in motion-related effects, a single RF frame was acquired for each measurement. Each recorded RF frame was a matrix in which the columns (57) represented the number of RF scanning lines in the acquisition window, while the rows (727) represented the number of samples in a single scanning line, with a sampling rate of 40 MHz. RF data were processed off-line using Matlab®.

Three quantitative parameters were extracted from the analysis of the RF signals, with the aim of finding differences between the healthy cartilage and the degenerated one, once treated with trypsin and collagenase: *(i)* the reflection index (RI), *(ii)* the thickness (d) and *(iii)* the phase entropy (sampEn). A region of interest (ROI) for all the samples was selected using a custom-designed Matlab® program, which allows to manually chose the start point of the ROI. A window composed of 15 scanning lines was selected in the central portion of each sample. The RF signal of each scanning line was then analyzed, calculating the parameters of interest in different segments of the RF signal related to different regions of the sample, as described below.

Reflection index (RI). RI was calculated at the first interface water -cartilage for all the RF lines (l = 15) included in the ROI, as described in equation (1):

$$RI = \frac{1}{l}\sum_{i=1}^{l} \max(RF_i) - \min(RF_i) \quad (1)$$

where l is the total number of RF lines (equal to 15) and RF indicates the RF signal for each scanning line. For each RF signal (727 samples), the first peak of reflection was identified on the squared RF signal, by using a threshold method with

respect to the baseline (*i.e.,* the signal from water). Then, the peak-to-peak value was calculated by identifying the maximum and minimum peak value in a window of 50 samples (corresponding to around 1 mm) starting from the first peak previously identified (see Figure 2a). Finally, the peak-to-peak values were averaged along the RF lines, thus obtaining one RI average value for each sample.

Thickness (d). The average thickness (d) of the cartilage tissue was calculated as in equation (2):

$$d = \frac{1}{l}\sum_{i=1}^{l} c_c \times \frac{TOF_i}{2} \qquad (2)$$

where TOF is the time of flight calculated from the cartilage top surface to the bottom of the sample, c_c is the speed of sound of the cartilage which was assumed 1610 m/s for the healthy cartilage, 1595 m/s after the trypsin treatment and 1580 m/s after the collagenase treatment [8]. The TOF was calculated by dividing the number of samples between the starting and the ending point of the cartilage (n_i in Figure 2b) by the sampling frequency of the system (40 MHz).

Phase entropy (sampEn). SampEn can be defined as the negative natural logarithm of the conditional probability that two sequences that are similar for m points, also match at the following point, according to equation (3) [14]:

$$sampEn(m, r, N) = -\log\frac{A}{B} \qquad (3)$$

where m is the length of the sequences, r is the tolerance for accepting matches, N is the length of the time series, whereas B and A are the probabilities that two sequences in the input are similar for m and m+1 points, respectively. In this study, the sampEn parameter was computed from the instantaneous phase signal in a selected ROI inside the sample with fixed dimensions (345 samples for each of the 15 scanning lines), setting the embedding dimension (m) to 3 and the tolerance (r) to 0.2 times the standard deviation of the original signal. Finally, a mean sampEn parameter for each sample was calculated by averaging the values along the RF lines (l=15).

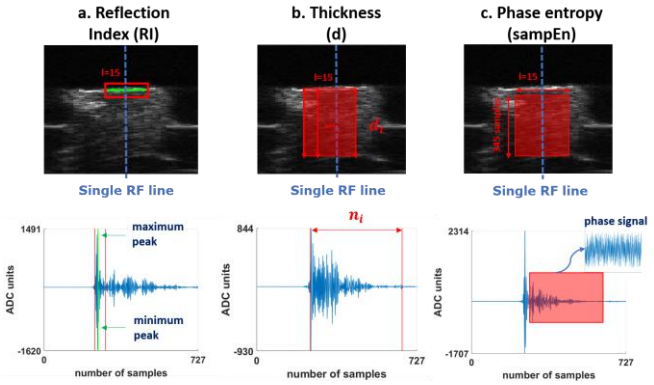

Figure 2 - Methods for the calculation of the quantitative ultrasound parameters used in this study (reflection index, thickness and phase entropy). For each parameter, a representative B-mode image with the selected ROI and an example of RF signal are shown. l= number of scanning lines (equal to 15 in our study), n_i= number of samples between the starting and the ending point of the cartilage for each scanning line (i), d_i= thickness of the sample for each scanning line (i).

III. Results

A. Morphology of cartilage samples

The SEM images shown in Figure 3 reveal the microscopic architecture of one representative sample for each experimental group: the control group (*i.e.,* the healthy cartilage) is shown in the left panel, the trypsin group is shown in the central panel and the collagenase group is shown in the right panel. After the collagenase treatment, the cartilage surface became much rougher and damaged with respect to the control group. In contrast, the effect of the trypsin treatment on the cartilage surface appeared to be a minor one.

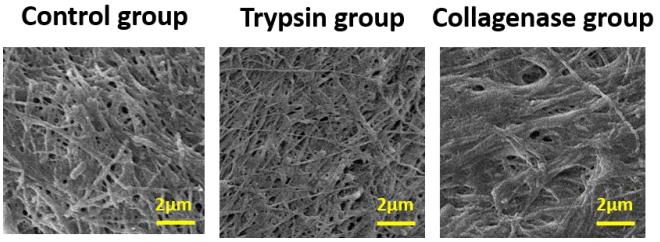

Figure 3 - SEM results. Micro-scale morphology of one representative sample for each experimental group.

B. Ultrasound data analysis

The three quantitative parameters (RI, d, sampEn) extracted from each group (control, trypsin and collagenase) were compared. Because of the non-normality of data distribution, the statistical comparison between control and treatment groups was performed using non-parametric Mann-Whitney tests for unpaired data using 0.05 as the level of significance.

In Figure 4a, the results for the RI parameter are reported. The reflection amplitude at the first interface decreased significantly in the case of samples treated with trypsin and collagenase, confirming a degradation effect on the cartilage surface.

Figure 4b shows the calculated d values. The cartilage thickness decreased significantly after collagenase digestion ($p<0.01$). Indeed, the thickness of the control samples was 9.29 ± 1.12 mm (median ± interquartile range, n=6), while the collagenase degraded samples had a thickness of 5.40 ± 1.50 mm. On the other hand, a non-significant decrease of the d value was observed after the trypsin treatment ($p>0.05$), namely 8.29 ± 1.65 mm.

In Figure 4c, the results obtained for the sampEn parameter are shown. The SampEn value increased after trypsin and collagenase treatment, thus indicating a higher irregularity in the instantaneous phase signal of RF data. However, the change was statistically significant only in the case of collagenase treatment.

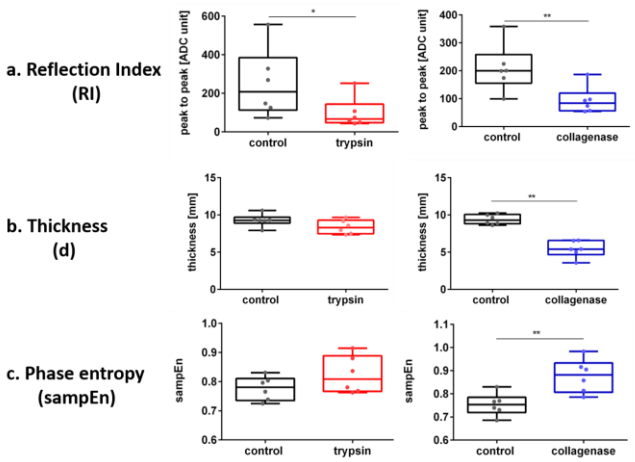

Figure 4 - Boxplot statistics of each parameter (reflection index, thickness and phase entropy) for healthy (control) and degraded cartilage (trypsin and collagenase) groups. *=p<0.05, **=p<0.01.

IV. Discussion and Conclusions

In this study, we demonstrated the possibility to use QUS parameters to detect changes in the status of bovine articular cartilage. This analysis was carried out using a transmission frequency equal to 15 MHz, which could be applied in future for an external (non arthroscopic) QUS diagnosis of OA. We investigated two parameters (*i.e.,* reflection and thickness) already explored in the literature, although at higher frequencies. In addition, we also explored a novel parameter in this context (*i.e.,* the phase entropy), which was effectively used for discriminating the main mineral bone components [11], but was never applied to cartilage.

We used two enzymatic degradation treatments (trypsin and collagenase), already used in the state of the art and acting on different cartilage components: collagenase is responsible for the collagen network degradation, while trypsin causes proteoglycan digestion [8]. It has been demonstrated that the collagen fibrils are the main reflectors of the US waves at the cartilage surface, while the proteoglycans have a marginal role in this regard [6]. Our results revealed that the reflection index dropped after both trypsin and collagenase digestion, even if the change was more significant in the case of collagenase treatment ($p<0.01$), as expected. This finding was confirmed by the SEM results presented in Figure 3: indeed, a degradation of the superficial collagen network was more evident after the collagenase treatment, rather than the trypsin one. Similarly, Nieminen *et al*. [15] assessed a significant decrease in the reflection coefficient after 4 hours of trypsin and 6 h of collagenase treatment using high-frequency US (29.4 MHz). Saarakkala *et al*. [6] found that the reflection coefficient dropped significantly only after 44 hours of collagenase digestion, using a transducer with a central frequency of 20 MHz, while no significant changes were found in the case of trypsin treatment. However, they performed the acoustic measurements after 60 min of trypsin digestion rather than 4 hours, as in our case. This suggests that also chemically digestion duration affects the results. Hence, the correlation of these results with morphological and/or histological analyses could be an important future perspective. Wang *et al*. [8] found that the integrated reflection coefficient decreased significantly after 24 hours of collagenase treatment, while no significant changes were found after 4 hours of trypsin action, using high-frequency US (40 MHz). However, the mathematical calculation of the reflection coefficient was performed in the spectrum domain rather than in the time domain, as in our case.

The cartilage thickness decreased significantly after the collagenase digestion (42%, $p<0.01$). On the other hand, no statistically significant thickness reduction was observed after the trypsin treatment (11%, $p>0.05$). The observed trend of the thickness parameters was in agreement with literature reports [8]. It is worth mentioning that in this study the thickness was calculated using reference values of speed of sound for healthy cartilage (1610 m/s), cartilage degraded with trypsin (1595 m/s) and cartilage degraded with collagenase (1580 m/s) [8]. In future experiments, the thickness could be directly measured (*e.g.,* using a caliber) to precisely evaluate changes in the speed of sound without any assumption.

Finally, we used SampEn to compute the entropy of the instantaneous phase of RF signals in a region within the samples. It has been recently demonstrated that the instantaneous phase derived from US signals can be correlated with the irregularities present in a scattering medium [16], thus giving information on the tissue microstructure. On the other hand, the SampEn is a measurement of the predictability of a time-series [14]. Previous studies investigated the entropy of the backscattered signal for the classification of tumors [17], for the assessment of hepatic steatosis [18] and fatty liver disease [19]. However, no studies applied the phase entropy of US signals for the assessment of healthy and degenerated cartilage tissues.

Hence, we used for the first time SampEn to quantify how the irregularities of the instantaneous phase series changed in response to enzymatic degradations of *ex-vivo* cartilage samples. The results in Figure 4, showed an increase in the SampEn parameter after both chemical treatments, even if the change was statistically significant only in the case of collagenase group ($p<0.01$). Such preliminary results suggest that the degradation action affects the instantaneous RF signal, increasing its randomness, probably due to enhanced inhomogeneities within the tissue structure. However, further experiments are needed to understand better the correlation between the phase entropy and the cartilage degradation level, also optimizing the protocol towards the *in-vivo* use. For example, SampEn could be combined with the reflection index in order to consider also the possible impact of US attenuation in the SampEn calculation.

It is worth mentioning that in this study we calculated the acoustic parameters without performing a normalization step with respect to a reference signal. Indeed, in all the previous studies concerning QUS for the diagnosis of tissue diseases, a reference signal was always used to normalize the tissue signal [4]. This is normally used to remove artifacts and dependencies from the acquisition systems. At a clinical level, this normalization step implies the use of a reference phantom with acoustic properties equal to the tissues of interest [10], thus resulting in a time-consuming and vulnerable to errors procedure. In our case, such a reference signal is not needed, with a clear advantage toward clinical translation.

Moreover, all the previous studies in this field performed the measurements through US transducers at very high frequencies (from 20 MHz to 55 MHz) [6] [5] [9]. Those frequencies can be translated to the clinical practice only

integrating these transducers within invasive arthroscopic probes. A substantial advantage of our study consists of the use of a certified clinical probe working in a frequency range normally used in the diagnostic echography of the joints (7.5 – 15 MHz), which could be applied externally to the patient, with a fully non-invasive procedure enabling frequent monitoring of the cartilage tissue conditions.

Further analyses are needed to better confirm these preliminary results and additional parameters (such as attenuation) could be investigated to discriminate healthy from degenerated cartilage. However, these findings confirm QUS as a powerful diagnostic tool for the *in-vivo* diagnosis of OA.

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