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# Biochemical similarity between cultured chondrocytes and *in situ* chondrocytes by chemometric analysis from FTIR microspectroscopy

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ABSTRACT

*Background aims:* Fourier Transform Infrared Micro-spectroscopy (FTIRM) is an emerging tool that obtains images with biochemical information of samples that are too small to be chemically analyzed by conventional Fourier transform infrared (FTIR) spectroscopy techniques. So, the central objective of this project was to study the biochemical similarity between articular and cultured chondrocytes by chemometric analysis from FTIRM.
*Methods:* Nine samples of knee articular cartilage were obtained; each sample was divided into two fragments, one portion was used for FTIRM characterization *in situ*, and from another part, chondrocytes were obtained to be cultured (*in vitro*), which were subjected to an FTIRM to characterize their biomolecular components. The FTIRM spectra were normalized, and the second derivative was calculated. From these data, principal component analysis (PCA) and a chemometric comparison between *in situ* and cultured chondrocytes were carried out. Finally, the biochemical mapping was conducted obtaining micro-FTIR imaging.
*Results:* FTIRM spectra of *in situ* and *in vitro* chondrocytes were obtained, and different biomolecules were detected, highlighting lipids, proteins, glycosaminoglycans, collagen, and aggrecan. Despite slight differences in the FTIR spectra, the PCA proved the organic similarity between *in situ* chondrocytes and cultured chondrocytes, which was also observed in the analysis of the ratios related to the degradation of the articular cartilage and collagen. In the same way, the ability of the FTIRM to characterize the molecular biodistribution was demonstrated.
*Conclusion:* The biochemical composition and biodistribution analysis using FTIRM have been useful for comparing cultured chondrocytes and *in situ* chondrocytes.


## 1. Introduction

Articular cartilage is an avascular, aneural, and alymphatic connective tissue designed to distribute the mechanical load and provide a wear-resistant surface to articulating joints [1]. Articular chondrocytes are the only type of cells present in cartilage of synovial joints, and play a crucial role in the production and maintenance of the extracellular matrix (ECM), once they are responsible for the synthesis of ECM, which is integrated by collagen type II, glycosaminoglycans (GAGs), and proteoglycans (PGs), mainly aggrecan (Fig. 1) [2]. The aggrecan, together with hyaluronan and link proteins, form high-molecular-weight aggregates that are necessary for the hydration of cartilage. The water-binding capacity is based on numerous anionic charges present in the long GAGs chains joined to the aggrecan [3].

*Abbreviations:* FTIRM, Fourier Transform Infrared Micro-spectroscopy; FTIR, Fourier Transform Infrared; ECM, extracellular matrix; GAGs, glycosaminoglycans; PGs, proteoglycans; OA, osteoarthritis; ACI, autologous chondrocyte implantation; SNV, standard normal variate; PCA, principal component analysis; FTIRI, Micro-FTIR images; MCT, Mercury-Cadmium-Tellurium.

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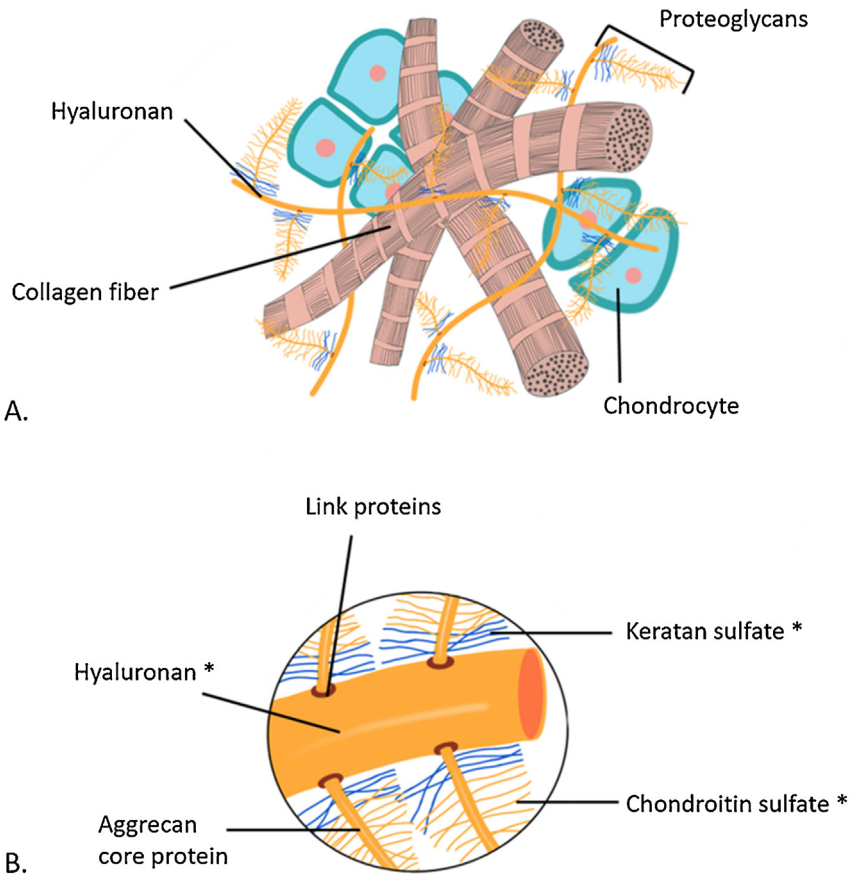


**Fig. 1.** Articular cartilage components. **A.** Chondrocytes and extracellular matrix integrated by collagen type II fibers, hyaluronan, and proteoglycans (PGs). **B.** PGs ultrastructure, PGs are united to hyaluronan by link proteins, the trunk of PGs is called aggrecan core protein, and two glycosaminoglycans (keratan and chondroitin sulfate) form the branches of the PGs. * GAGs (glycosaminoglycans).

Half of the world's population aged 65 years or older, has osteoarthritis (OA), which is the most prevalent disorder of articulating joints in humans [4]. The phenotype of this disease results from some possible abnormalities of the connective tissue combined with aberrant chondrocyte behavior and with an overwhelming of the cartilage reparative abilities [1]. The OA is characterized by the progressive destruction of the cartilage that lines joints, the subchondral bone surfaces, and synovium, which carries pain, immobility, muscle weakness, and reduction in function and the ability to complete activities of daily living. Aging is an important OA risk factor due to its association with the accumulation of advanced glycation end products in articular cartilage [5].

For more than two centuries, it has been known that traumatic defects of the articular cartilage do not heal spontaneously. Therapeutic interventions for the treatment of these defects aim to delay or prevent progression to OA [6]. Nowadays, there is not a cure for this debilitating disorder; nevertheless, new therapies are emerging, such as bioengineered matrices [7], and a promising cell-based repair strategy known as autologous chondrocyte implantation (ACI), which has been introduced in clinics.

To develop an ACI process, firstly, the chondrocytes are extracted from their native environment of the cartilage tissue and are grown in monolayer cultures for its amplification until an adequate number of cells are obtained [6].

Nevertheless, it is known that cells in culture tend to differentiate. So, there is a need to characterize cultured chondrocytes before implantation to use these cells as cell therapy. In this sense, molecular and phenotypic techniques have been used for cellular characterization such as PCR, RT-qPCR, immunocytochemistry, flow cytometry, among others. However, the mRNA analysis in cartilage tissue is a big problem for two reasons; firstly, the transcripts of collagen type II and aggrecan are large molecules, and their genes contain several introns that are sequenced relatively late. Secondly, the cell density in cartilage is very low, leading to little yields in RNA isolation. Moreover, the highly anionic GAGs of PGs interfere with the extraction of nucleic acids, also decreasing the yield of RNA isolation [3].

Besides, some of those techniques consume too much cell sample and provide little morphological information, without giving cellular information about molecular localization in the tissue, highlighting that these techniques are expensive and need specialized personnel [8,9]. For this reason, new techniques to evaluate the chondrocytes biochemical profile have been thought.

As commented in previous works [10,11], Fourier transform infrared (FTIR) spectroscopy and Fourier transform ion cyclotron resonance could result in powerful tools in the study and diagnostic of biological systems (as cultured cells), once the combination with the standard (protein expression) and computational-chemometrical methods is possible.

FTIR spectroscopy is a technique that can provide fundamental information about the molecular structure of organic and inorganic materials. One of the significant advantages of this technique is its versatility and its sample-saving property. In the FTIR analysis, absorption of IR radiation occurs when a photon is transferred to a molecule and excites it to a higher energy state; after that, this excited state results in the vibrations of molecular bonds generating a variability of wavenumbers or frequencies [12]. In biological materials, different biomolecules can be detected, such as lipids, proteins, carbohydrates, and nucleic acids, which have a unique chemical structure and thus distinctive IR spectra.

However, developments in infrared sources, optics, and detectors over the past decade have led to the marriage of FTIR spectroscopy with light microscopy, enabling spectroscopic microscopy within the complex environment of biological cells and tissues [13].

FTIR micro-spectroscopy (FTIRM) is an emerging tool that obtains images with its biochemical information; this technique has become popular to characterize samples that are too small to be chemically analyzed by conventional FTIR techniques. So that, it has been employed to analyze different components in histological specimens, allowing the analysis of a single cell, detecting chemical bonds in cells or tissues that can then be related to the presence and composition of biomolecules such as lipids, DNA, glycogen or collagen [14].

The coupling of FTIRM and visible light microscopy opens the possibility of the visualization and mapping of functional group and molecular arrangements in samples, resulting a mapping measurement, where the infrared spectrum at each sampling point is measured and integrated; moreover, parameters like peak areas in defined spectral regions are used to map the distributions of target functional groups [12]. This method has been dubbed "chemical photography," once semi-quantitative information can be obtained from direct consideration of spectra or, using standard image processing software from the pictures themselves [15].

The advances in FTIRM for tissue imaging have been driven in large part by three key developments: 1) increased speed of data acquisition due to the availability of large detectors which typically have thousands of IR sensitive detectors, 2) development of advanced processing algorithms and computational power to handle large hyperspectral data sets, and 3) modelling of FTIR imaging systems to maximize spatial resolution [14].

So far, our research group has performed the morphological, genetic, and phenotypic comparison between human articular chondrocytes (*in situ*) and cultured chondrocytes (*in vitro*) by optic microscopy, RT-qPCR, and immunofluorescence, confirming the non-dedifferentiation of the cells [16]. However, these techniques consume the entire sample and much time. So, due to the advantages of FTIRM and its chemical mapping, the central objective of this project was to study the biochemical similarity between articular and cultured chondrocytes by chemometric analysis from FTIR microspectroscopy.

## 2. Materials and methods

### 2.1. Study population

Nine samples of 10 $mm^2$ of healthy articular cartilage were obtained from the non-load area of the joint surface (lateral condyle), considering the full depth of the cartilage. Each sample was divided into two fragments, one portion was used for FTIRM characterization, and from another portion, articular chondrocytes were obtained by enzymatic disaggregation to be cultured and expanded *in vitro*.

### 2.2. Sample collection

Written informed consents for the obtention of cartilage sample, cell expansion, and characterization were obtained. The Institutional Human Research Ethical Committee approved the protocol and the informed consent forms. All experiments were examined and approved by the appropriate ethics committee and had therefore been following the ethical standards laid down in the 1964 Declaration of Helsinki.

For this study, ten patient females and males aged between 20 and 45 years arrived at the orthopedics department of the Hospital Central Militar undergoing to a diagnostic or therapeutic knee arthroscopic procedure in the period from December 2016 to June 2017, were invited to participate in this research. Nine patients agreed to participate and completed the study.

### 2.3. Chondrocytes culture

The sample portion designated to cell culture was minced into small fragments of approximately 2–3 $mm^3$. Subsequently, as described in previous works [16], the samples were desegregated enzymatically employing 0.25% trypsin solution (Invitrogen; 140349) and 3% penicillin-streptomycin (10,000 IU/ml–10,000 μg/ml) (Invitrogen; 15140) for 30 min, and 2 mg/ml collagenase (Sigma; C0130) for 8 h.

Once digestion process was completed, no digested fragments were eliminated and the obtained cell solution was centrifuged at 1500 rpm for 5 min getting a cell pellet, which was resuspended and seeded at a density of $10^4$ cells/$cm^2$, using DMEM supplemented with 15% Fetal Bovine Serum (FBS) (ATCC; 30-2020), 1% penicillin-streptomycin (10,000 IU/ml–10,000 μg/ml) (Invitrogen; 15140), and 2 mM l-alanine-l-glutamine (ATCC; 30-2115). Culture dishes were incubated at 37 °C in a humidified incubator (5% $CO_2$, 95% air). Cell culture medium was replaced every four days until reaching 21 days in culture.

### 2.4. FTIR micro-spectroscopy

Cartilage tissue sections of each patient and cell smears from the cultured chondrocytes of each patient were analyzed by FTIRM using an FTIR microscope (Jasco; IRT-5200) coupled to an FTIR spectrometer (Jasco; 6600), measuring each sample in triplicate.

For which purpose, articular cartilage samples (*in situ* chondrocytes) were embedded in tissue-tek (Sakura cat. 4583) and frozen; after that, cryosections of 5 μm were obtained and mounted on a gold-coated microscope slide with a gold layer thickness of 100 nm (Aldrich; 643246-5EA).

On the other hand, for the cultured chondrocytes (*in vitro* chondrocytes) analysis, the cells were centrifuged at 1200 rpm for 3 min and then washed twice employing isotonic salt solution. The supernatant was detached in each wash; after that, 3 μl of the cell suspension were placed on the surface of a gold-coated microscope slide, making a cell smear. Once the samples were mounted on the gold-coated slide, a single chondrocyte was focused and dried at room temperature for about 15 min to remove excess water, measuring the spectra until the absorption bands related to water were undetectable employing a Cassegrain objective of 32 × . Each spectrum was collected in the mid-infrared range (4000-400 $cm^{-1}$) at a spectral resolution of 4 $cm^{-1}$ with 120 scans.

### 2.5. Spectral treatment

Once the FTIRM spectra (crude spectra) were acquired, a standard normal variate (SNV) normalization treatment was applied using the software Unscrambler X version 10.3 (Camo software AS); the normalized and averaged spectra were plotted using the Origin 6.1 software (OriginLab). Subsequently, the second derivative spectra were calculated using the Savitzky-Golay algorithm, which applies a successive adjustment of adjacent point data subsets with a low polynomial degree by least squares to the linear approximation.

### 2.6. Multivariate analysis

From the averaged FTIRM spectra of each patient, the second derivative was calculated, which were used as input data for the application of the multivariate method known as principal component analysis (PCA) using the software Minitab version 13.2 (Minitab Inc.). The analyzed regions corresponded to amide I

(1715-1588 $cm^{-1}$), GAGs (1390-1362 $cm^{-1}$), collagen type II (1357-1325 $cm^{-1}$), and aggrecan (1145-1036 $cm^{-1}$).

### 2.7. Chondrogenic comparison by spectral analysis

The main chondrogenic components, such as amide I, GAGs, collagen type II, and aggrecan [17], as well as the ratios of amide I/amide II and collagen type II/amide II related to the degradation of the articular cartilage and collagen respectively [18], were calculated and compared in both groups.

Regarding amide I, GAGs, collagen type II, and aggrecan, the integrated areas were assessed in the spectral regions of 1715-1588 $cm^{-1}$, 1390-1362 $cm^{-1}$, 1357-1325 $cm^{-1}$, and 1145-1036 $cm^{-1}$ respectively. On the other hand, the ratios of amide I/amide II and collagen type II/amide II were calculated as follows: A1715-1588 $cm^{-1}$ / 1588-1479 $cm^{-1}$ and A1357-1352 $cm^{-1}$ / A1715-1588 $cm^{-1}$, respectively.

### 2.8. Biochemical mapping

Micro-FTIR images (FTIRI) were collected on the FTIR microscope fitted with a liquid nitrogen-cooled MCT (Mercury, Cadmium, Tellurium) detector, coupled to an FTIR spectrometer, using a 32X Cassegrain objective. Microscope optics permitted to amplify the image 1:2. The absorbance spectra were acquired in reflectance mode at a spectral resolution of 4 $cm^{-1}$ with 80 scans coadded. Biochemical images were obtained by automated mapping of multiple points (IQ mapping) of the FTIR microscope. The analyzed biomolecules were lipids (1747-1737 $cm^{-1}$), amide I (1675-1624 $cm^{-1}$), GAGs (1376 $cm^{-1}$), collagen type II (1338 $cm^{-1}$), and aggrecan (1140-984 $cm^{-1}$). The analysis of each spectral band was represented in a two-dimensional image, which reconstruction corresponded to the density distribution of each chemical species within the specimen.

## 3. Results

### 3.1. FTIR micro-spectroscopy analysis

Before FTIRM analysis, cultured chondrocytes were morphological, protein, and genetically validated, as cited in prior works [16]. As previously mentioned, all the samples were analyzed by FTIRM in reflectance mode. Fig. 2 shows the normalized crude FTIRM spectra, where absorption bands associated with different types of biomolecules such as lipids, proteins, GAGs, collagen, and PGs are detected, showing differences in structural and biochemical composition between *in situ* and *in vitro* chondrocytes.

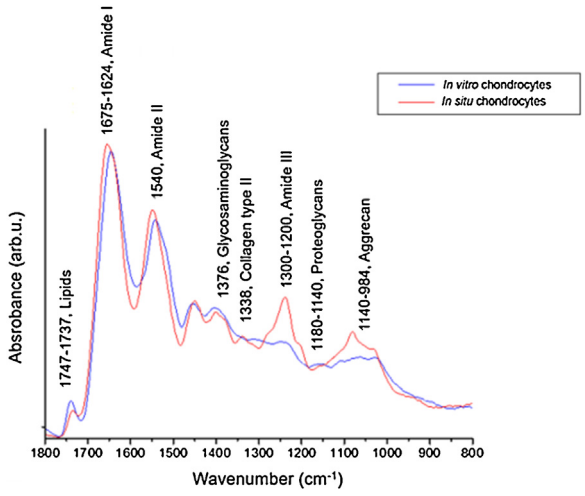


**Fig. 2.** FTIR spectra of *in vitro* and *in situ* chondrocytes measured in the fingerprint region (1800–800 $cm^{-1}$). Several absorption bands of lipids, proteins, glycosaminoglycans, collagen type II, and proteoglycans are observed. Each spectrum corresponds to the average of nine samples, which were measured three times.

Firstly, at the range between 1747-1737 $cm^{-1}$, absorption bands associated with the C = O stretching mode of lipids esters are observed, where a higher band intensity is displayed in the *in vitro* chondrocytes. After that, the absorption bands between 1715-1588 $cm^{-1}$ and the peak at 1540 $cm^{-1}$ correspond to the functional groups amide I (C = O stretching) and amide II (C-N stretching + N-H bending) of the proteins respectively; in these bands, a shift and greater absorbance intensity in the spectrum of the *in situ* chondrocytes are shown. Subsequently, the absorption bands at 1376 $cm^{-1}$ are related to $CH_3$ symmetric bending vibrations of GAGs, where higher band intensity is displayed in the *in vitro* chondrocytes. The next bands at 1338 $cm^{-1}$ are related to the $CH_2$ side-chain vibrations and the triple helical structure of collagen type II. Afterward, the spectral interval between 1300-1200 $cm^{-1}$ is associated with the collagen amide III vibrations with a significant mixing with $CH_2$ wagging vibration from the glycine backbone and proline side chain; this band showed higher intensity in the spectrum of the *in situ* chondrocytes. The next absorption bands between 1180-1145 $cm^{-1}$ are related to C-O stretching vibrations of the carbohydrate residues corresponding to PGs. Finally, the absorption bands at the range of 1145-1036 $cm^{-1}$ are associated with the C-O stretching vibrations of the carbohydrate residues in collagen and PGs, mainly the aggrecan [17,19], where a greater band intensity is showed in the *in vitro* chondrocytes.

### 3.2. Principal component analysis

To correlate the multiple variables obtained from the second derivative spectra by FTIRM, a PCA was performed in the amide I, GAGs, collagen type II, and aggrecan regions (Fig. 3). The first two depicted components (PC1, PC2) explain 76.1%, 99.2%, 99.6%, and 95.4% of the correlation between both groups in these regions, respectively.

### 3.3. Biochemical chondrogenic comparison

Regarding the chondrogenic components, amide I, GAGs, collagen type II, aggrecan, as well as the ratios related to the degradation of the articular cartilage (amide I/amide II) and collagen (collagen type II/amide II) respectively, similar amounts of the aforementioned components were observed in both study groups (Fig. 4). No statistical significance was found between the groups in any analysis.

### 3.4. IQ mapping analysis

The cellular localization of specific cartilage biomolecules such as lipids, amide I, GAGs, collagen type II, and aggrecan in the *in vitro* chondrocytes was developed using the IQ mapping function. Fig. 5A shows the visible image of a single chondrocyte. The total absorbance images from FTIRI are presented in Figs. 5B-5 F. Biochemical maps of lipids, amide I, GAGs, collagen type II, and aggrecan are shown. Each image represents the integrated absorbance of a specific band of the IR spectra for each pixel of the MCT detector; red and blue colors represent strong and weak absorption respectively of the infrared beam. According to the aforementioned, the highest area of absorbance for lipids is inside the cell, in the cytoplasmic area (Fig. 5B); on the other hand, amide I absorbance appears to be concentrated in the ECM proximal to the chondrocyte, and in less intensity in the cellular membrane of the chondrocyte (Fig. 5C). In the same way, GAGs (Fig. 5D), collagen

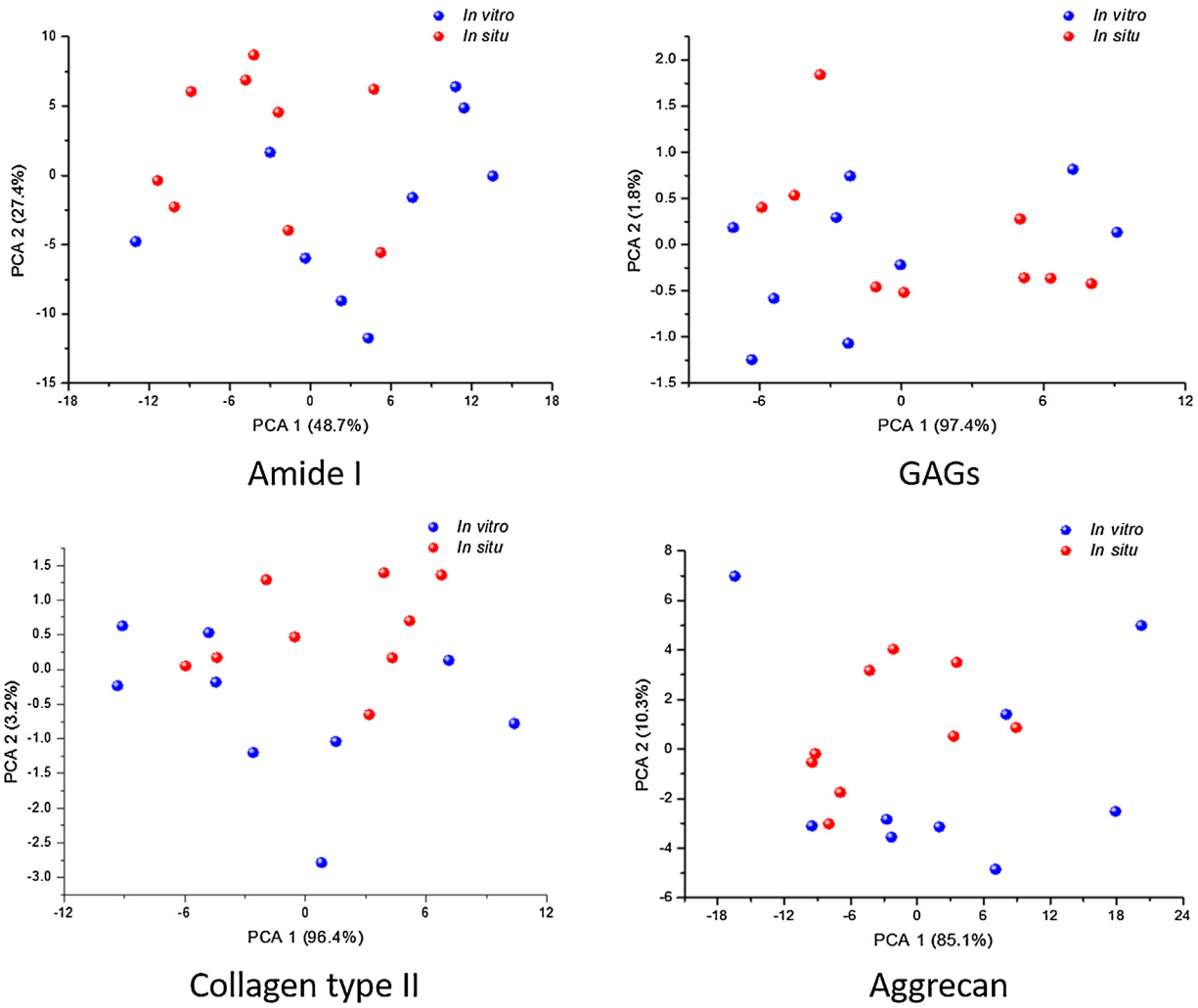


**Fig. 3.** Principal components analysis (PCA) of *in vitro* and *in situ* chondrocytes in the regions: **A.** Amide I, **B.** Glycosaminoglycans, **C.** Collagen type II, **D.** Aggrecan.

type II (Fig. 5E), and aggrecan (Fig. 5F) showed a higher absorbance in the ECM than inside the cell.

## 4. Discussion

The importance of autologous cultured chondrocytes for their subsequent use as a therapeutic option in regenerative medicine for joint injuries has been mentioned [16,20]. Likewise, the technique of an adequate culture of autologous chondrocytes has been demonstrated through its genetic, protein, and morphological characterization. Unfortunately, these analyses consume the majority of the sample, are laborious and expensive, reason by which, a fast and sample-saving technique for its biochemical characterization using FTIRM and its biochemical mapping function has been raised, techniques that nowadays have not been reported in the literature for this ending.

Despite similarities of biochemical profiles between *in situ* and *in vitro* chondrocytes in the FTIRM spectra, changes in intensities and frequencies of some absorption bands were evidenced, particularly in the protein and aggrecan spectral regions, suggesting structural and functional differences, which could be attributed to mitotic and metabolic activity of the cultured chondrocytes, that has been reported previously [16].

Regarding the biochemical analysis, the spectra obtained in the biological fingerprint region of *in situ* and *in vitro* chondrocytes correspond to biological samples, which also correspond to cartilage tissue, observing bands of biomolecules such as proteins, GAGs, collagen type II, and PGs, mainly aggrecan [21]. These results agree with Yin and Xia (2011), who studied the chemical and structural distributions of cellular components surrounding individual chondrocytes in canine humeral cartilage [22], reporting bands related to amide I, amide II, amide III, collagen type II, and carbohydrates. However, in our FTIRM spectra, we could perceive a band related to lipids, which was not identified by Yin and Xia, although it is important to mention that they analyzed canine humeral articular cartilage using ATR-FTIRM, whereas we analyzed human knee chondrocytes. In the same way, our results are similar to those reported by Rieppo et al. (2017), who investigated human articular cartilage [17], although they neither reported a lipids band.

About lipids band (1747-1737 $cm^{-1}$), a higher intensity in the spectrum of the *in vitro* chondrocytes was observed, which is related to a higher expression of this biomolecule in these cells; about this, it is known that human chondrocytes express several proteins for fatty acid metabolism and cholesterol biosynthesis. These molecules are up-regulated during chondrogenesis, indicating a high cholesterol biosynthesis in these cells. Cholesterol biosynthesis is required for normal growth plate chondrogenesis in rats. In the same way, Villalvilla et al. (2013) stated that cholesterol signaling stimulates the hypertrophy of in vitro chondrocyte through nuclear receptor retinoid-related orphan receptor-α (Ror-α) expression, and it is also necessary for apoptosis protection

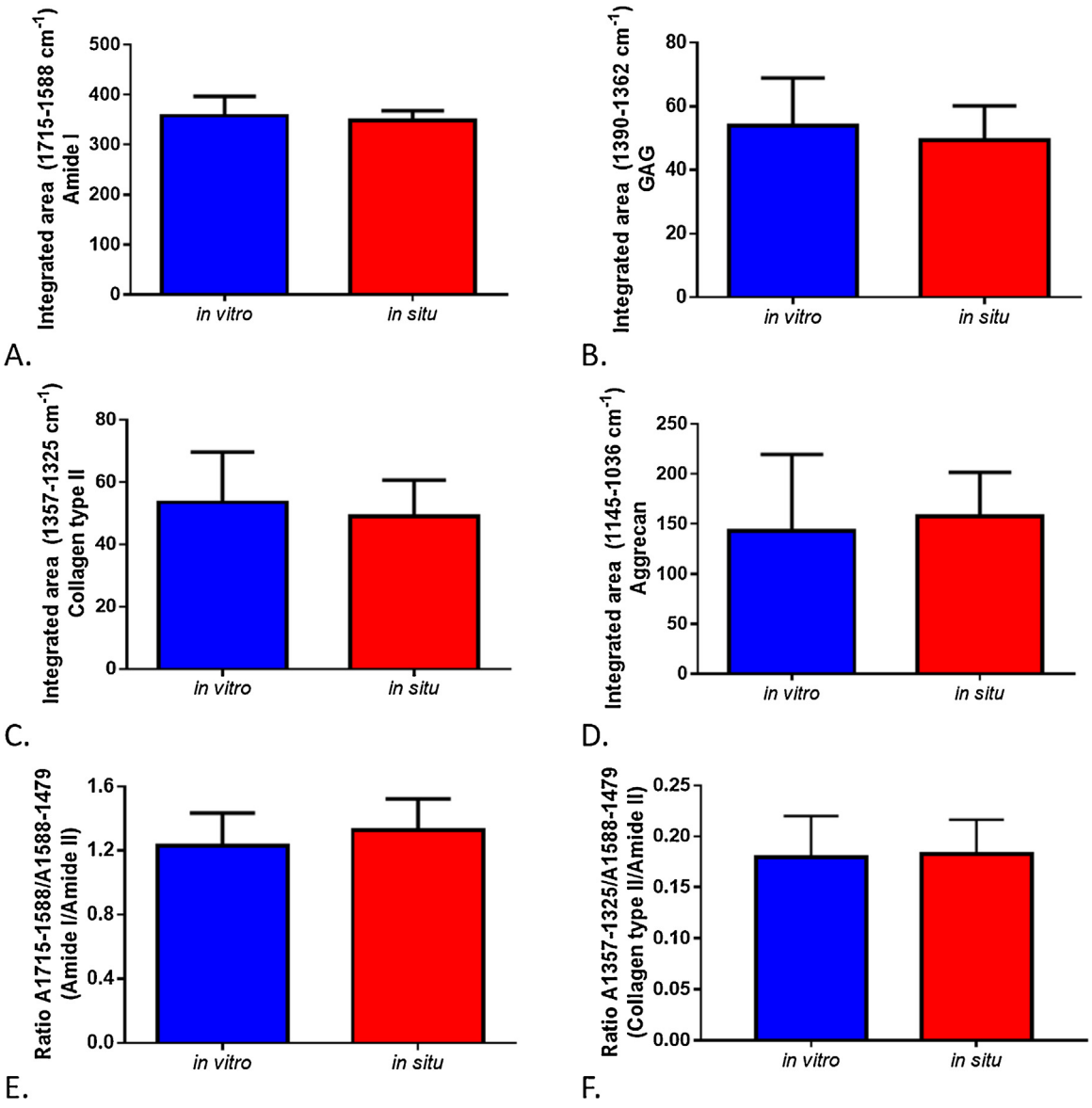


**Fig. 4.** Chondrogenic comparison of *in vitro* and *in situ* chondrocytes by chemometric spectral analysis of the main articular components (integrated areas) and the ratios related to the degradation of the articular cartilage and collagen. **A.** Amide I, **B.** Glycosaminoglycans, **C.** Collagen type II, **D.** Aggrecan, **E.** Amide I / Amide II, **F.** Collagen type II / Amide II. No statistical significance was found between the groups in any analysis.

during chondrogenesis and growth plate development [23]. So that, in the spectrum of the *in vitro* chondrocytes, a higher concentration of lipids were detected by FTIRM, which could be related to the *in vitro* growth of these cells. With all those mentioned above, we can state that Rieppo et al., and Yin and Xia did not detect lipids due to they analyzed cartilage *in situ*, and these cells are not growing.

Concerning the band amide I (1715-1588 $cm^{-1}$), Mandair and Morris (2015) have reported that the most widely reported collagen band is amide I [24]. In the same way, Rieppo et al. have stated that this band is attributed to mature collagen [17], and Camacho et al. (2001) reported that this band is associated with the amount of collagen in the ECM [25]. In our results, this band presented a higher absorbance in the spectrum of *in situ* chondrocytes, probably due to the ECM of these chondrocytes was not digested. So, the amount of collagen is more significant in the *in situ* chondrocytes than in the *in vitro* chondrocytes, which were subjected to a digestion process employing collagenase. Moreover, regarding FTIRI, as expected, the major concentration of this biomolecule was detected in the ECM. Additionally, these results are strengthened with the lower absorbance of the amide II band at 1540 $cm^{-1}$ in the spectrum of the *in vitro* chondrocytes. According to Rieppo et al., this band is correlated with the integrity of the collagen so that once cultured chondrocytes were subjected to an enzymatic digestion process, the peak related to amide II decreased in the spectrum of the *in vitro* chondrocytes [17].

Respect to the GAGs band at 1376 $cm^{-1}$, it is known that this biomolecule includes hyaluronan, keratan sulfate, and chondroitin sulfate (Fig. 1) [3], which are essential components of the ECM. In the spectrum of the *in vitro* chondrocytes a widening of this band and an increase absorbance was observed, which could be related to GAGs synthesis augment, linked to cell proliferation mainly in the G1 phase [26]; besides, these biomolecules are essential for the smooth functioning of articular cartilage [27]. Regarding FTIRI, the results corresponded to the expected one, showing a greater concentration of GAGs in the ECM.

About collagen type II band (1338 $cm^{-1}$), it is known that the synthesis of this biomolecule is characteristic of chondrocyte

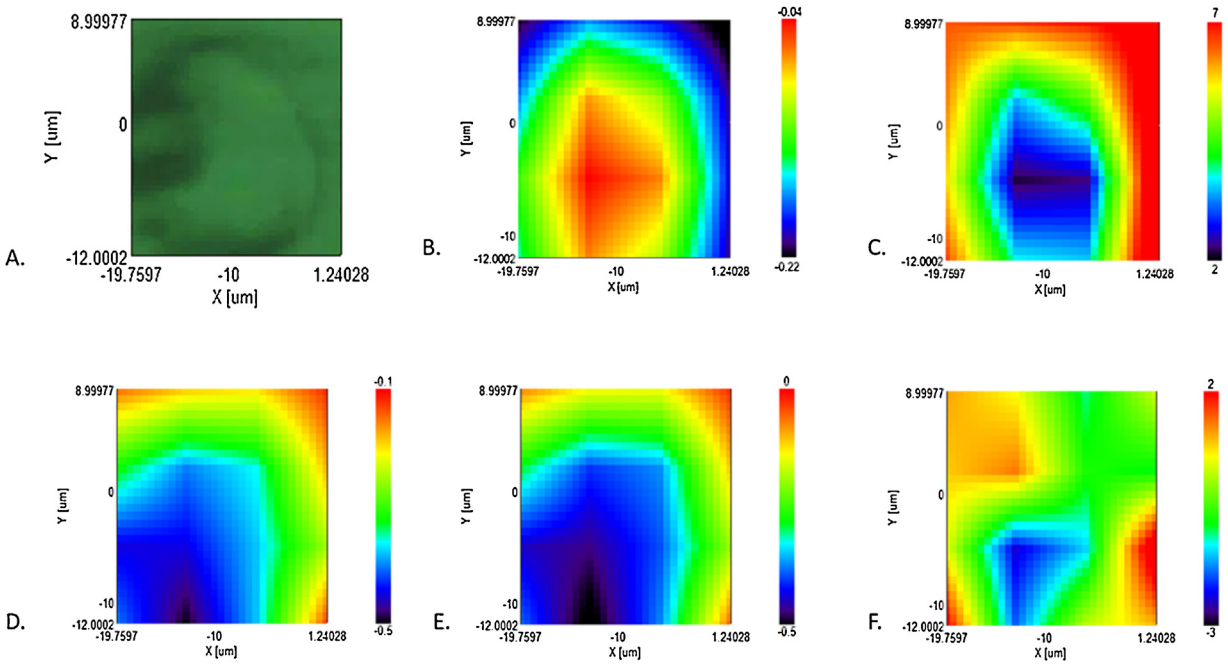


**Fig. 5.** FTIRI of a single *in vitro* chondrocyte, showing the biochemical map of different biomolecules and its biodistribution. **A.** Visible image of the chondrocyte. **B.** Lipids (1747-1737 $cm^{-1}$), **C.** Amide I (1675-1624 $cm^{-1}$), **D.** Glycosaminoglycans (1376 $cm^{-1}$), **E.** Collagen type II (1338 $cm^{-1}$), **F.** Aggrecan (1140-984 $cm^{-1}$). Red and blue colors represent strong and weak absorption of the infrared beam.

lineage cells [6]; moreover, collagen is the most abundant structural macromolecule in the ECM; specifically, collagen type II represents from 90% to 95%, and forms fibrils and fibers intertwined with PGs aggregates [28]. As mentioned previously, Rieppo et al. have associated this band in conjunction with amide II to the collagen integrity [17], so that, the spectrum of the *in vitro* chondrocytes showed a lower absorbance than the spectrum of the *in situ* chondrocytes. Moreover, the FTIRI showed the collagen biodistribution quite similar to GAGs distribution, once GAGs are intertwined with the collagen fibers.

On the other hand, it is known that the PGs are a type of glycoprotein heavily glycosylated, and in the articular cartilage, they represent the second-largest group of macromolecules in the ECM, accounting from 10% to 15% of the wet weight [23,28]. Articular cartilage contains a variety of PGs that are essential for normal function, including aggrecan, decorin, biglycan, and fibromodulin, among others [29,30]. The band related to this biomolecule (1180-1145 $cm^{-1}$) showed a greater absorbance in the spectrum of the *in vitro* chondrocytes, probably due to this biomolecule is associated with GAGs, and as mention previously, the band related to GAGs was also higher in cultured chondrocytes.

Moreover, according to Rieppo et al., the band associated with carbohydrates region between 1145-1036 $cm^{-1}$, is correlated with the amount of aggrecan in pure compound mixtures of this component [17]. It is known that the largest proteoglycan in size and the most abundant by weight is the aggrecan, which possesses more than 100 chondroitin sulfate and keratan sulfate chains, and is characterized by its ability to interact with hyaluronan forming large proteoglycan aggregates via link proteins [28]. In our results, we can observe that there is a higher absorbance of this band in the spectrum of the *in situ* chondrocytes, probably due to the conservation of ECM; therefore, in the FTIRI results, a higher concentration of this biomolecule in the ECM was also observed.

On the other hand, respect to the calculation of the ratios related to the degradation of the articular cartilage (amide I/amide II) and collagen (collagen type II/amide II), similar amounts of these components were observed, and no statistical significance was found between the groups in any analysis, which could be related to the conservation of the molecular characteristics after cell culture.

Despite the slight differences in the FTIR spectra, the PCA proved the organic similarity between *in situ* and cultured chondrocytes, which was also observed in the ratios analysis related to the degradation of the articular cartilage and collagen. Considering all those mentioned above, we demonstrated that there are no significant differences between the molecular components of *in situ* and cultured chondrocytes, which makes the cultured chondrocytes feasible for it use in medical procedures, once these chondrocytes retain the morphology and chemometric of an in situ chondrocyte.

## 5. Conclusions

The biochemical composition and biodistribution analysis using FTIRM and its biochemical mapping function, have been useful for comparing cultured chondrocytes and *in situ* chondrocytes, consuming a smaller amount of the sample compared to the classic techniques such as immunocytochemistry and PCR assays, among others. Nevertheless, trials with other cell lineages and tissues are required to strengthen this biological application in regenerative medicine.

## Data Availability

All the generated data and the analysis developed in this study are included in this article.

## Funding

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## Declaration of Competing Interest

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.btre.2019.e00391.

## References

[1] A. Mobasheri, S.J. Vannucci, C.A. Bondy, S.D. Carter, J.F. Innes, M.F. Arteaga, et al., Glucose transport and metabolism in chondrocytes: a key to understanding chondrogenesis, skeletal development and cartilage degradation in osteoarthritis, Histology and histopathology 17 (2002) 1239–1267.
[2] N. Okumura, S. Imai, F. Toyoda, E. Isoya, K. Kumagai, H. Matsuura, et al., Regulatory role of tyrosine phosphorylation in the swelling-activated chloride current in isolated rabbit articular chondrocytes, The journal of physiology 587 (2009) 3761–3776.
[3] M.J. Lammi, J. Häyrinen, A. Mahonen, Proteomic analysis of cartilage- and bone associated samples, Electrophoresis 27 (2006) 2687–2701.
[4] D. Xing, Y. Xu, Q. Liu, Y. Ke, B. Wang, Z. Li, J. Lin, Osteoarthritis and all-cause mortality in worldwide populations: grading the evidence from a meta-analysis, Scientific Reports 6 (2016) 24393.
[5] S. Yamabe, J. Hirose, Y. Uehara, T. Okada, N. Okamoto, K. Oka, et al., Intracellular accumulation of advanced glycation end products induces apoptosis via endoplasmic reticulum stress in chondrocytes, The FEBS journal 280 (2013) 1617–1629.
[6] A. Tekari, R. Luginbuehl, W. Hofstetter, R.J. Egli1, Chondrocytes expressing intracellular collagen type II enter the cell cycle and coexpress collagen type I in monolayer culture, Journal of orthopaedic research 32 (2014) 1503–1511.
[7] E.A. Noyszewski, K. Wroblewski, G.R. Dodge, S. Kudchodkar, J. Beers, A.V. Sarma, et al., Preferential incorporation of glucosamine into the galactosamine moieties of chondroitin sulfates in articular cartilage explants, Arthritis and rheumatism 44 (2001) 1089–1095.
[8] T. Nolan, R.E. Hands, S.A. Bustin, Quantification of mRNA using real-time RT-PCR, Nature protocols 1 (2006) 1559–1582.
[9] J.S. Makki, Diagnostic implication and clinical relevance of ancillary techniques in clinical pathology practice. Clinical medicine insights, Pathology 9 (2016) 5–11.
[10] M.M. Mata-Miranda, G.J. Vázquez-Zapién, M. Rojas-López, V. Sánchez-Monroy, D.G. Pérez-Ishiwara, R.J. Delgado-Macuil, Morphological, molecular and FTIR spectroscopic analysis during the differentiation of kidney cells from pluripotent stem cells, Biological Research 50 (2017) 14.
[11] G.J. Vázquez-Zapién, M.M. Mata-Miranda, V. Sánchez-Monroy, R.J. Delgado-Macuil, D.G. Pérez-Ishiwara, M. Rojas-López, FTIR spectroscopic and molecular analysis during differentiation of pluripotent stem cells to pancreatic cells, Stem Cells International 2016 (2016) ID 6709714.
[12] Y. Chen, C. Zou, M. Mastalerz, S. Hu, C. Gasaway, X. Tao, Applications of micro-Fourier Transform Infrared spectroscopy (FTIR) in the geological sciences – A review, International journal of molecular sciences 16 (2015) 30223–30250.
[13] L.M. Miller, P. Dumas, From structure to cellular mechanisms with infrared microspectroscopy, Current opinion in structural biology 20 (2010) 649–656.
[14] H. Sreedhar, V.K. Varma, P.L. Nguyen, B. Davidson, S. Akkina, G. Guzman, et al., High-definition Fourier Transform Infrared (FT-IR) spectroscopic imaging of human tissue sections towards improving pathology, Journal of visualized experiments: JoVE 95 (2015) 52332.
[15] F. Palombo, S.G. Cremers, P.D. Weinberg, S.G. Kazarian, Application of Fourier transform infrared spectroscopic imaging to the study of effects of age and dietary L-arginine on aortic lesion composition in cholesterol-fed rabbits, Journal of the Royal Society, Interface 6 (2009) 669–680.
[16] M.M. Mata-Miranda, C.M. Martínez-Martínez, J.E. Noriega-González, L.E. Paredes-González, G.J. Vázquez-Zapién, Morphological, genetic and phenotypic comparison between human articular chondrocytes and cultured chondrocytes, Histochemistry and cell biology 146 (2016) 183–189.
[17] L. Rieppo, J. Töyräs, S. Saarakkala, Vibrational spectroscopy of articular cartilage, Applied spectroscopy reviews 52 (2017) 249–266.
[18] A. Boskey, N.P. Camacho, FT-IR imaging of native and tissue-engineered bone and cartilage, Biomaterials 28 (2007) 2465–2478.
[19] L. Rieppo, S. Saarakkala, T. Närhi, H.J. Helminen, J.S. Jurvelin, J. Rieppo, Application of second derivative spectroscopy for increasing molecular specificity of Fourier transform infrared spectroscopic imaging of articular cartilage, Osteoarthritis and Cartilage 20 (2012) 451–459.
[20] M.M. Mata-Miranda, L.E. Paredes-González, C.M. Martínez-Martínez, G.J. Vázquez-Zapién, Implante de condrocitos autólogos con potencial regenerativo en lesiones articulares, Revista Colombiana de ortopedia y traumatologia 29 (2015) 28–35.
[21] Y. Zhang, F. Wang, H. Tan, G. Chen, L. Guo, L. Yang, Analysis of the mineral composition of the human calcified cartilage zone, International journal of molecular sciences 9 (2012) 353–360.
[22] J. Yin, Y. Xia, Chemical visualization of individual chondrocytes in articular cartilage by attenuated-total-reflection Fourier Transform Infrared Microimaging, Biomedical optics express 2 (2011) 937–945.
[23] A. Villalvilla, R. Gómez, R. Largo, G. Herrero-Beaumont, Lipid transport and metabolism in healthy and osteoarthritic cartilage, International journal of molecular sciences 14 (2013) 20793–20808.
[24] G.S. Mandair, M.D. Morris, Contributions of Raman spectroscopy to the understanding of bone strength, BoneKEy reports 4 (2015) 620.
[25] N.P. Camacho, P. West, P.A. Torzilli, R. Mendelsohn, FTIR microscopic imaging of collagen and proteoglycan in bovine cartilage, Biopolymers 62 (2001) 1–8.
[26] S.F. Preston, C.S. Regula, P.R. Sager, C.B. Pearson, L.S. Daniels, P.A. Brown, et al., Glycosaminoglycan synthesis is depressed during mitosis and elevated during early G1, The Journal of cell biology 101 (1985) 1086–1093.
[27] J. Jerosch, Effects of Glucosamine and Chondroitin Sulfate on Cartilage Metabolism in OA: Outlook on Other Nutrient Partners Especially Omega-3 Fatty Acids, International Journal of Rheumatology 2011 (2011)969012.
[28] A.J. Sophia Fox, A. Bedi, S.A. Rodeo, The basic science of articular cartilage structure, composition and function, Sports health 1 (2009) 461–468.
[29] E. Hedbom, P. Antonsson, A. Hjerpe, D. Aeschlimann, M. Paulsson, E. Rosa-Pimentel, et al., Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage, The Journal of biological chemistry 267 (1992) 6132–6136.
[30] J. Xu, D. Mosher, Fibronectin and other adhesive glycoproteins. The extracellular matrix: an overview, in: R.P. Mecham (Ed.), Biology of extracellular matrix, Springer-Verlag, Berlin-Heidelberg, Germany, 2011, pp. 41–75.