Molecular analysis of the destruction of articular joint tissues by Raman spectroscopy

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Abstract

Introduction: Osteoarthritis (OA) is a highly heterogenous disease influenced by different molecular, anatomic and physiologic imbalances. Some of the bottlenecks for enhanced diagnosis and therapeutic assessment are the lack of validated biomarkers and early diagnosis tools. In this narrative review, we analyze the potential of Raman spectroscopy (RS) as a label-free optical tool for the characterization of articular joint tissues and its application as a diagnosis tool for OA.

Areas covered: Raman spectra produce a unique “molecular fingerprint” providing rotational and vibrational molecular information, allowing the identification and follow-up of molecular changes associated with OA pathological mechanisms. Focusing on multiple joint tissues (cartilage, synovium, bone, tendons, ligaments and meniscus) and their contribution in disease incidence and progression, this review highlights the current knowledge on the application of RS in the characterization of organic and inorganic molecules present at these tissues and alterations that occur in the onset of OA.

Expert Opinion: Vibrational spectroscopy techniques, such as RS, are low cost, rapid and minimally invasive approaches that offer high specificity in the assessment of the molecular composition of complex tissues. Combined with multivariate statistical methods, RS offers great potential for optical biomarkers discovery or disease diagnosis applications, and we hereby discuss clinical translational progresses on the field.

1. Introduction

Osteoarthritis (OA) is a chronic and multifactorial disease affecting 50% of the population over 65 years, being one of the main causes of disability in the aged population [1]. OA is a disorder involving the movable joints characterized by cell stress and extracellular matrix

degradation, initiated by micro/macro-injuries that activate maladaptive repair responses. The disease initially manifests as a molecular derangement followed by anatomic and/or physiologic imbalances that culminate in illness [2]. Being highly heterogenous, OA can be classified in different phenotypes with people presenting very different clinical manifestations and outcomes. Moreover, the presence of multiple joints and tissues in the musculoskeletal system complicate disease incidence and progression. Nowadays, there is no cure and even though drugs available alleviate pain and clinical therapies help halting or minimizing joint damage progression, there are still unmet needs to deliver more efficient treatments, such as validated biomarkers or improved early diagnosis techniques [3,4].

One of the main limitations for OA early diagnosis lies on the onset of the disease itself, as in its initial phases it is asymptomatic and when patients attend physicians, they present charateristics above the subclinical threshold [2]. After patient history review and physical examination [5], OA clinical diagnosis is confirmed by joint radiography, the gold standard technique, or magnetic resonance imaging (MRI). Severity of OA can then be classified according to Kellgren-Lawrence (K-L) grading system, between 0 (healthy) and IV (severe OA) grades [6–8]. This indirect classification method identifies changes in the joint bone tissue, osteophytosis and joint space narrowing, but is insufficient to detect small changes in cartilage [9]. On the other hand, MRI allows the analysis of surface and depth lesions, as well as the visualization of pre-radiographic alterations in multiple tissues or intra-articular bodies, for which a MRI-based OA definition is available [10,11]. However, application of a strong electromagnetic field invalidates this method for patients with metal implants or other medical devices. In addition, MRI routine use for OA diagnosis would be unrealistic for public health systems due to high costs [12,13]. Other method for articular damage assessment is arthroscopy, which detects chondropathies in early OA, but image analysis is subjective and leads to a certain degree of intervariability [14]. Moreover,

cartilage degradation evaluation can be complemented with histomorphometric evaluations, such as the Kraus' modified Mankin Score or the histopathology OARSI (Osteoarthritis Research Society International) system [15].

Given the limitations of conventional techniques for OA diagnosis and its increasing prevalence it is paramount to develop non- or minimally-invasive techniques, capable of detecting damage or changes at the molecular level. Biochemical biomarkers have emerged as promising tools with enhanced sensitivity and reliability regarding the imaging techniques available. These could facilitate early diagnosis of joint destruction, disease prognosis and monitoring of progression through biochemical tests [4]. Even though significant advances have been made due to proteomic tools using blood serum, synovial fluid or cell secretome, up to now, only candidate biomarkers have been described related to pathological mechanisms of OA [3,16,17].

Raman spectroscopy (RS) is an emerging biomedical technology in the study of diseased tissues and could offer significant improvements for the early diagnosis for a variety of musculoskeletal diseases, including OA, and the definition of optical biomarkers [18,19]. RS is based on the inelastic photonic dispersion that occurs when a monochromatic light beam hits a molecule, providing rotational or vibrational modes information. A given Raman spectrum represents specific peaks or bands at a certain frequency or wavelength, constituting a well-defined "molecular fingerprint" of the tissue analyzed (Figure 1) [20]. Raw data will then be typically preprocessed in order to overcome baseline interference and may also involve peak normalization [21,22]. Thereafter, data can be classified according to unsupervised or supervised statistical methods, allowing sample classification (biomarkers) or diagnostic analysis, where the latest involves the use of additional information obtained from gold standards, such as histopathology or radiography scores. Importance should also be given to the impact of inter- and intra-variability when using the

aforementioned scores [23]. Moreover cross-validation against biochemical tools is frequently included [24].

Due to alternative processing pathways, data is not always suitable for direct comparison. Nonetheless, mean Raman spectra and the corresponding algorithm-based extracted data can be interpreted and discussed in the context of Raman shifts corresponding to biological and/or biochemical assignments in cartilage or surrounding tissues. Protocol standardization is thus fundamental in order to overcome limitations of RS for cartilage tissue analysis, such as high fluorescence or weak signal strength inherent to most biological samples that influence spectral quality and detection accuracy [25].

In addition, RS presents other advantages, such as time and cost of analysis efficience [24], as it can be performed directly on a very small volume of sample and it does not suffer from interference from water molecules. Other vibrational spectroscopic techniques, i.e., Fourier transform infrared (FTIR), and near infrared (NIR) spectroscopies can also be combined with Raman, enabling assessment of quantitative and qualitative features of articular cartilage, which have been reviewed elsewhere [26].

The main goal of this narrative review was to analyse available data on RS for the characterization of cartilage and other articular joint tissues that present alterations during OA progression, shedding light on its potential for OA diagnosis and optical biomarkers.

2. Raman Spectroscopy for OA diagnosis

2.1. Redefining articular cartilage characterization

The analysis of articular cartilage by RS can be found in the literature from 2006 onwards, including studies using different human joint tissues and/or animal model validation (Table 1). Eventhough RS allows a non-invasive analysis of cartilage, most references describe studies with sample pre-treatment, such as paraffin, formalin, poly(methyl methacrylate) (PMMA) or cryomatrix, from which signal contribution can be easily identified in the spectra, although caution should be taken when comparing to fresh tissue [18,19,27–30]. In

addition, as lasers can be coupled to microscopes, it is possible not only to study alterations in superficial cartilage but also in transversal sections.

Articular cartilage is a connective tissue formed by chondrocytes [31], embedded in an extracellular matrix (ECM), composed by collagens, mainly type-II (Col-II) [32], proteoglycans (PGs), lipids and other minority non collagenous proteins. PGs are composed of protein units of aggrecan linked to hyaluronan fibers, constituing a protein core that connects one or more glycosaminoglycan (GAGs) chains. Due to cartilage composition, Raman spectra are complex, being predominantly dominated by the presence of bands associated with the aforementioned matrix proteins.

Dehring et al. [33], early described articular cartilage molecular composition using RS, in a mice model. A molecular fingerprint of articular cartilage is depicted in Figure 2A and the assignments corresponding to the different bands related to cartilage components, type and bond mode are detailed in Table 2. Briefly, the main bands that have been associated with proteins are as follows: the doublet 856-880 cm^{-1}, related to the distribution of proline/hydroxyproline (Pro-Hyp), the amino acids band, at 1004 cm^{-1} [34], related to phenylalanine [19,27], commonly used as a normalization band [28], and the doublet at 1245-1270 cm^{-1} (amide III), related to the secondary structure of proteins and their relative distribution [35]. In addition to the bands associated with collagen, cartilage spectra reveals a peak at 1063 cm^{-1}, corresponding to sulfated GAGs, with a predominant contribution of chondroitin sulfate (CS) [18,27,28,34,36] and other specific bands related to GAGs, such as the pyranose ring, at 1039-52 cm^{-1} [34]. When mineralized tissue is present, interferences at 1063 cm^{-1} have been reported, due to an overlapping carbonate band, at 1070 cm^{-1}. Alternatively, 1375 cm^{-1} has been suggested to study PG alterations [36]. Moreover, in overdamaged tissues where the subchondral bone is exposed, an additional band can be identified at 958 cm^{-1}, attributed to phosphate minerals [27]. More recently, Mansfield and Winlove [30] studied lipids distribution, identifying two related

regions: a band at 1441 cm^{-1} and 2845-2930 cm^{-1} displacements.

The first study using RS for detecting joint damage in human cartilage has established ratios for tissue degradation, such as cartilage-to-bone ratio (1063/958), phosphate to collagen ratio (958/920) (a measure of mineral/organic ratio), and carbonate to phosphate ratio (1070/958) (indicating the degree of carbonate substitution in hydroxyapatite crystals), corresponding to molecular events described during OA progression [27]. These events were associated with collagen degradation and disorganization, already observed in early stages, and PGs content abrupt decrease in more advanced stages of the disease [37]. These can also be related to mineralization processes, as nucleation and crystal deposition of basic calcium phosphate (BCP) around collagen fibers are favoured with GAGs decrease [38]. In addition, recent studies have described an indirect lipid index (IL, 1450/1668) which increases with OA radiological progression (K-L grade) [39]. Some of these molecular changes can be observed in Figure 2B that depicts RS spectra from human cartilage tissue derived from patients with different K-L grades. Given the pivotal role of cartilage mechanical properties, Lim et al., used a porcine model to study healthy cartilage subjected to different loads. The authors demonstrated that GAGs-related bands decreased in impact groups, indicative of PG loss, which was also confirmed by histomorphological analysis. Moreover, variations in the Pro-Hyp ratio and the shift of amide III band to a doublet of peaks were both related to changes in collagen structure. Variables such as cartilage thickness affected RS results, as observed by the presence of a phosphate band, at 957 cm^{-1}, in impact groups-only that correlated with a decrease in cartilage thickness and thus greater laser penetration into deeper layers [34].

Bergholt and coworkers developed a RS-image analysis method based on mapping intensities of specific signals across the surface of transversal sections of bovine cartilage. Their goal was to study depth-dependent relative composition and collagen fibers' orientation within cartilage zonal arquitecture [29]. By complementing RS with multivariate

analysis, they established and validated six clearly defined and differentiated zones, in contrast with the commonly accepted four histological areas (superficial, intermediate, deep zone and subchondral bone). Later, a biochemical quantification of these zonal gradients supported the reliability of this technique to detect depth-dependent arrangements [40]. Recently, the authors offered a comprehensive review of RS for cartilage extracellular matrix (ECM) and other tissues, discussing further challenges in biomedical applications [41]. Raman confocal microscopy studies were also carried out at single-cell level using isolated chondrocytes of patients with different International Cartilage Repair Society (ICRS) grades that have shown a concomitant decrease in protein and nucleic acid levels and an increase of lipid deposits with disease progression [42].

2.2. Synovium

The synovial membrane is a connective tissue that lines the inner side of the diarthrodial joints, tendon sheaths and joint pockets [43]. It produces the synovial fluid (SF), composed of an ultrafiltrate of blood supplemented with additives, produced by synoviocytes. Its function is to nourish cartilage and lubricate joints for which lubricin, a glycocomposite binded to hyaluronan (HA), plays a major role [44–46]. During OA, synovial fluid proteins become progressively denatured [47]. Plasma infiltration induces a decrease in HA concentration and molecular weight, caused by abnormal metabolic processes [48]. These changes result in the loss of SF lubricating properties and viscosity [44,49]. In addition, there is an increase in inorganic crystals and cytokines that can also cause synovitis or inflammation of the synovial membrane [50].

SF aspirates are currently been studied using proteomics and mass spectrometry approaches, defining different profiles of proteins, metabolites or other substances, for the diagnosis of rheumatic diseases, including OA [51–54]. Even though it is not possible to extract information from precise proteins using Raman spectra, joint damage could be

predicted by analyzing variations of HA in deposited droplets of SF. Dehring et al., designed a SERS method, based on the analysis of artificial SF, for HA quantification, validated under detection limits appropriate to its' concentration range in OA synovium [48]. As a decrease in HA is associated with a loss of SF viscoelastic properties, in the onset of OA, it could constitute a potential biomarker for the early stages of the disease. Other authors have examined human SF derived from knee OA patients, using combined SERS and resonant Raman spectra, being able to discriminate low- from high-grade groups with a classification accuracy of 100% [55].

Another study with SF from healthy and radiographic joint damage patients (K-L: 0-I vs II-IV) reported a significant decrease in proteins with alpha-helix structure, entailing alterations in the chemical environment of the protein backbone, in OA patients [56]. This could be related to other observations on the macromolecular level, such as the release of Col-II fragments from adjacent tissues [57]. Furthermore, an association between SF droplets' morphology and OA damage has been suggested. In one hand, joint damage was weakly correlated with the presence of ferm-shaped crystals in SF droplets' core, whilst a moderate correlation was found with the presence of radial cracks at the droplets' edge. When spectra was obtained from different regions in the droplet, significant results were found amongst different ratios, related to protein bonds (1080/1001 cm^{-1}, 1080/1125 cm^{-1} and 1655/1448 cm^{-1}) and their relative secondary structure (1235/1260 cm^{-1} and 1670/1655 cm^{-1}) [57]. RS has also been used to detect crystalline deposits in SF, from urate and calcium - basic calcium phosphate (BCP) or calcium pyrophosphate dihydrate (CPPD), with potential for diagnosing other rheumatic diseases, such as gout and pseudogout [58–61]. Some of these compounds have also been suggested as indicators of joint space reduction that occurs during OA progression [62].

2.3. Bone

Bones constitute the basic anatomical structure in the human body around which other musculoskeletal tissues are arranged. Bone is formed by organic and inorganic components, varying in proportion depending on the type of bone, age, diet and disease processes [63–65]. In contrast to cartilage, the most abundant collagen is from type-I (Col-I) and bone mineral is constituted of a carbonated form of a nanoscrystalline nonstoichiometric apatite [66,67]. RS has been extensively applied for bone properties assessment and characterization, summarized elsewhere [68,69]. The most characteristic assigned bands for bone, by RS are thus, those related to the mineral components, specifically hydroxyapatite phosphate (v_1 PO_4^{3-}, v_2 PO_4^{3-} and v_3 PO_4^{3-}) and B-type carbonate (v_1 CO_3^{2-}) (Table 2). Nonetheless, even though bone mineral apatite consists mainly of B-type carbonate, it has been reported the presence of A-type carbonate (1114 cm^{-1}) in older bones [69]. Additionally, nonspecific signals related to the organic fraction of the tissue (amide III doublet and amide I), can be found [27,70]. Other parameters have also been covered, such as mineral to matrix ratio, mineral maturity/crystallinity, relative carbonate content or relative tissue water content/porosity [71].

During OA progression, alterations in the subchondral bone occur due to bone overgrowth, a process known as osteophytosis [6]. Also, OA patients suffer from a decrease in bone quality and microhardness making them more susceptible to bone injuries [72]. Several RS studies reported differences in bone tissues from healthy and OA patients [70,73]. A concomitant decrease in hydroxyapatite to collagen ratios (960/1244 cm^{-1} and 960/1268 cm^{-1}) and an increase in carbonate to phosphate ratio (1071/960 cm^{-1}) would most likely be related to compensatory mechanisms in response to a mineralization deficit. In addition, an increase in collagen secondary structure disarrangement (1268/1244 cm^{-1}) indicated a disorganization of collagen in the bone matrix. These alterations were found in the subchondral bone derived from OA femoral heads. However, when analyzing cancellous bone, which lays directly under the subchondral bone, significant differences were not

found [70]. In more recent studies, two-dimensional Raman maps were used to calculate the degree of bone mineralization (DBM) in cancellous and subchondral bone from femoral heads, by integrating hydroxyapatite' signal relative intensity to that of collagen [63]. The results were consistent with a decrease in the subchondral bone mineralization, although it is not yet fully supported that this parameter can provide an accurate diagnosis of OA by itself. Kerns et al., performed a similar study in tibial plateau, reporting bone matrix chemistry changes for OA tissues [74].

2.4. Tendons and ligaments

Tendons and ligaments are a type of fibrous connective tissue present in the joints. Tendons and ligaments are mainly composed of Col-I, so their spectra are dominated by bands of this component (Table 2). Other non-collagenous proteins, lipids and proteoglycans can be found in a very low percentage, and thus have a limited overall contribution, with the additional limitation that their corresponding bands overlap with those of collagen [66,75].

Frushour and Koening [76], took the first step in studying the composition of tendons by RS. The authors identified bands at 1248 and 1271 cm^{-1}, related to amide III vibrations. The most relevant signals found in tendons, were those of amide I, at 1665 cm^{-1}, the CH_2/CH_3 deformation, at 1457-64 cm^{-1}, the amide III envelope, at 1220-1280 cm^{-1}, and the C-C bound (Pro-Hyp), at 820-80 cm^{-1} [77,78]. These assignments were used as references for identifying variations in collagen backbone or fibers orientation, under tension conditions, that support RS application for determining tension in ligaments and tendons [79,80]. Later, this technology was suggested to monitor mechanical properties of ligaments, such as tensile stress, during knee surgery, promoting better ligament fixation, by setting a proper tension in a repaired or grafted tissue, contributing to a normal kinematics of the knee [81]. Moreover, analysis of supraspinatus tendon enthesis spectra

led to the identification of a mineralization gradient associated to mineral crystallinity across the tendon-bone insertion site [82–84].

During OA, both tendons and ligaments become progressively weaker and inflammatory processes can be triggered. Ligament degradation or displacement is usually related with disease progression [85–87] and ligament instability may produce changes in tension distributions that can accelerate the disease [88]. So far, only one study has demonstrated an increase in ligament stiffness in OA patients [89], although no references were found for the application of RS in the evaluation of tendons and ligaments during OA.

2.5. Meniscus

The meniscus is a structure present at the knee joint and its main role is load distribution. It is a fibrocartilaginous tissue with Col-I and -II and GAGs as main components [90]. When meniscus' function is altered due to its deterioration, imbalance in load distribution causes damage to both cartilage and the subchondral bone, inducing OA [91]. During disease progression, meniscal tissue undergoes variations in its mechanical properties [92,93]. Moreover, at the biochemical level, collagen fibers become disorganized and tissue calcifications appear [94,95].

Levillain et al. [91], used RS to study changes in the meniscus of rabbits in an early OA model. The main Raman signals identified in the meniscus were those related with proteins, such as proline (857 cm^{-1}), hydroxyproline (877 cm^{-1}), phenylalanine (1004 cm^{-1}), amide III (1220-1280 cm^{-1}), C-H organic bond (1447-1452 cm^{-1}) and amide I (1666 cm^{-1}). Disarrangement of collagen networks, as well as an increase in GAGs, in response to degradation, were observed in histological stainings, although these were not evident in Raman spectra, suggesting there could be limitations in assessing early OA stages. Moreover, mineralization assessment of the meniscus was previously performed in advanced OA tissue, identifying hydroxyapatite (960 cm^{-1}) and CPPD deposits (1049 cm^{-1}) [96].

3. RS for OA candidate biomarkers

According to this literature review, Figure 3 represents a selection of bands and ratios that could constitute a panel of biomarkers for OA, considering changes in the different tissues present in the joint. Briefly, in cartilage, proline to hydroxyproline (856/880 cm^{-1}) and random coil to α-helix secondary structure (1245/1270 cm^{-1}) ratios could be involved with changes in collagen synthesis or randomness, respectively, and bands at 1063, 1040, and 1375 cm^{-1} could be related to proteoglycans relative content. Other ratios could be considered to assess cartilage quality and tissue mineralization processes, such as cartilage to bone (1063/958 cm^{-1}), bone to collagen (960/920 cm^{-1}), and carbonate to phosphate (1070/958 cm^{-1}) ratios [27]. Some of these ratios have been lately validated against K-L grade, Mankin Score and biochemical assays [97]. Concomitantly, changes in bone during OA could be detected by hydroxyapatite to collagen (960/1245 cm^{-1} and 960/1270 cm^{-1}), carbonate to phosphate (1070/960 cm^{-1}) and collagen randomness (1270/1245 cm^{-1}) ratios. Regarding SF, a candidate for liquid biopsy, proposed bands are the following: 899, 940, 1050, 1130 cm^{-1}, as well as 1410 cm^{-1}, to identify HA, previously suggested as an OA biomarker [48]. In addition, protein (1080/1001 cm^{-1}, 1080/1125 cm^{-1} and 1655/1448 cm^{-1}) and collagen structure ratios (1235/1260 cm^{-1} and 1670/1655 cm^{-1}) also offer potential. Finally, in the meniscus, the main biomarkers that could indicate tissue damage would be those related to calcium deposits (960 cm^{-1} and 1049 cm^{-1}).

4. Conclusions

Raman spectroscopy is a promising technique for the detection and assessment of musculoskeletal joint tissues molecular composition with high clinical significance for the diagnosis of osteoarthritis. In this narrative review, we have highlighted the contribution of RS in detecting and characterizing alterations in organic and inorganic molecules, present in the multiple joint tissues, which occur in the onset of OA. Future steps include in one hand, overcoming technical limitations and on the other, defining and validating a panel of

optical biomarkers based on variations on the molecular fingerprint of healthy cartilage and/or complemented with those from surrounding tissues, which are also affected during this multifactorial and heterogenous disease.

5. Expert Opinion

Osteoarthritis imposes great challenges for the treating physician. Despite significant socioeconomic burden, existing resources are still insufficient for an early diagnosis. The heterogeneity of the disease and a considerable variability in the trajectory of its prognosis, with some individuals experiencing progression whilst others remain stable for several years, has led to the consideration of OA as a syndrome comprised of multiple and distinct phenotypes rather than a single disease. Even though efforts have been made to identify clinically relevant OA phenotypes, there is still a lack of standardization [98,99]. Due to its high molecular specificity and the possibility of a fast and economic monitoring, RS could help to better define patients' subgroups.

RS is currently being tested in clinical settings to measure bone transcutaneously and has been assessed in an early stage clinical trial for osteoporosis, OA and osteogenesis imperfecta (ClinicalTrials.gov Identifier: NCT02814591). Most Raman instruments are compatible with fiberoptic probes, which could be used in different approaches, through arthroscopes during arthroscopic surgery or in needle arthroscopes available in ambulatory offices [100]. For instance, fiber-optic RS for arthroscopy has been tested in a proof of principle study for cartilage-bone analysis in human individuals, in exposed tissues [27], supporting its suitability for medical environment. More recently, a similar approach, has been developed using infrared fibre probe coupled to an arthroscope [101]. These studies are thus paving the way for the clinical translation of fiberoptics that could differentiate heterogeneties in cartilage, as a complementary tool to the clinic [27,41]. Another translational application regarding cartilage is the application of RS to detect and

monitor ochronotic and non-ochronotic tissues, a clinical feature of alkaptonuria rare disorder [102].

6. Five-year view

As novel approaches towards understanding the progression of OA are being made, the discovery and validation of biomarkers will support better managing disease progression and improve prognosis. As such, RS is a powerful source for the obtainance of label-free optical biomarkers giving new insights in the molecular basis of OA and providing reliable biomolecular information for diagnosis and treatment evaluation, as it is being considered for other diseases [102–104]. Indeed, as explored in this review, several joint tissues have been independently evaluated, identifying specific signals that could constitute candidate biomarkers (Figure 3), yet these need further analytical validation. New paradigms can also have an impact on the focus given in RS applications for OA diagnosis. For example, according to current literature, strong evidence has demonstrated that bone changes are not simply a secondary sign of OA, but can be an initiating factor of degeneration of the joint, suggesting changes in the molecular structure of the subchondral bone may precede cartilaginous changes in the OA joint [105]. Some advances have already been made to shed light on the role of bone in OA using RS. Kerns and coworkers have shown preliminary results on early alterations in bone matrix changes in the subchondral bone of control and OA tibial plateaus [106] and another study in femoral heads revealed that OA subchondral bone is hypermineralised, supported by an increase in the phosphate-to-amide I ratio and a decrease in carbonate substitution into apatite crystals [107].

In addition, technical limitations still need to be overcome, such as tissue autofluorescence, low signal intensity, or possible phototoxic effects after prolonged exposure to the laser. Several solutions are currently being explored that involve the use of low energy NIR lasers (λ=785, 830 and 1064 nm), the optimization of parameters suchs as laser power or exposure time that can minimize the signal-to-noise ratio, or establishing

standardized protocols that involve mathematical and computational modeling in spectra data processing and analysis [22]. Indeed, the implementation of more advanced multivariate or machine learning approaches is being explored under unsupervised and supervised algorithms. For example, k-means clustering of full cartilage spectra has been suggested as an exploratory technique to group similar spectra and can elucidate into samples that have similar or different composition [38]. Also, a recent study applied k-means clustering of selected signals/ratios (960/1004 cm^{-1}, 1063/1004 cm^{-1} and 1245/1270 cm^{-1}) of spectra obtained from human OA cartilage, derived from femoral condyle and head, which led to the definition of two clusters, corresponding to a low and a high severity profile, validated against K-L grade and histological Mankin Score (MS). Based on this, a predictive model, using receiver operating characteristic (ROC) curves, has been explored, setting up specific thresholds, being able to discriminate patients with high OA severity (K-L≥III and MS≥9) with a probability of more than 80% [108]. Finally, considering that OA affects different joints, most frequently knee, hip, hands and the spine, but also being increasingly seen in ankles and feet, it is expected that due to the flexibility of Raman spectroscopy technology, the number of studies will continue to increase.

7. Article Highlights

- Raman spectroscopy is able to successfully discriminate between healthy and osteoarthritic joint tissues.

- Raman spectra provide detection and monitoring of cartilage molecular alterations at different stages of OA.

- A panel of Raman signals and ratios assigned to cartilage tissue components can be related to specific events that occur during tissue destruction and thus are proposed as OA optical biomarkers.

- RS has high potential as a rapid and economic method for OA early diagnosis.

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Figures and Tables List:

Figure 1. Schematic representation of Raman spectroscopy analysis setting of cartilage *ex vivo* explants.

Figure 2. A. Typical Raman spectrum of human healthy (H) cartilage ("molecular fingerprint") obtained with a 1064 nm laser excitation (v: stretching mode, δ: deformation mode). Spectra was background corrected for clarity of presentation. B. Raman spectra for H and OA human cartilage (K-L grades: 0 to IV).

Figure 3. Schematic representation of tissues present in the knee joint and suggested optical biomarkers for OA (characteristic bands or ratios and main modifications involved) according to the literature.

Table 1. Raman spectroscopy methods and sample characteristics in the analysis of changes in the molecular composition of cartilage in the included studies.

Table 2. Raman spectroscopy assigned signals and corresponding groups for each tissue present in human joints.