5. Raman spectroscopy for cartilage damage severity, degradation and repair assessment.

P. Casal-Beiroa[1, 2], F.J. Blanco[1, 2,3], J. Magalhães[1, 2,4]

[1]Unidad de Medicina Regenerativa. Grupo de Reumatología (GIR). Instituto de Investigación Biomédica de A Coruña. Complejo Hospitalario Universitario de A Coruña (CHUAC).

[2]Centro de Investigaciones Científicas Avanzadas (CICA), Universidade da Coruña (UDC).
[3]Departamento de Medicina, Facultad Ciencias de la Salud, Campus de Oza, Universidade da Coruña (UDC).

[4]Centro de Investigación Biomédica en Red (CIBER). Madrid, España

joana.cristina.silva.magalhaes@sergas.es

Osteoarthritis (OA) is the most common rheumatic disease, characterized by progressive cartilage degradation. Current gold-standard diagnosis present limitations for detecting early stages of the disease, apart from being subjective to the reader. Raman spectroscopy (RS) has been recently described as a label-free, non-invasive tool to detect chemical and molecular changes in cartilage, producing a unique fingerprint of the tissue. This technique presents high potential as early diagnosis tool, promoting the discovery of optical biomarkers. Moreover, due to its specificity, RS could also be applied as a tool to monitor clinical outcomes as well as preconditioning of cell- or scaffold-based regenerative medicine therapies for cartilage repair and OA.

Introduction

Chronic pathologies that affect the joint tissues represent one of the major causes of disabilities in the elder population, being osteoarthritis (OA) one of the most common musculoskeletal diseases, affecting the hip, knees, hands, feet and spine (Blanco, 2014, Musumeci et al., 2015). Even though there are several clinical therapies currently being applied to patients, there are still several unmet needs for successful diagnosis and treatment, such as, biomarkers discovery and validation, patient phenotype definition and more efficient early diagnosis and/or follow-up tools (van der Kraan et al., 2016, Karsdal et al., 2016, Deveza et al., 2017).

OA severity is generally determined by the widely accepted gold standard Kellgren-Lawrence (K-L) radiographic score albeit it presents limitations for OA early-stage diagnosis (Kellgren and Lawrence, 1957, Altman et al., 1986). MRI whole joint-based assessment of knee OA has also been proposed (Hunter et al., 2011), however MRIs' high cost and incompatibility for some patients, limits its application as a regular medical care practice. On the other hand, assessment systems such as Mankin Histological-Histochemical Grading System (Mankin et al., 1971) and others (Pearson et al., 2011, Pascual Garrido et al., 2009), commonly used for cartilage degradation assessment during different stages of OA development, are time consuming, complex and subjective to the reader (Pauli et al., 2012) (Fig. 5.1).

Raman Spectroscopy (RS), a type of vibrational spectroscopy based on the inelastic scattering of photons, is a non-destructive technique that provides a chemical specific signature, commonly referred to as "molecular fingerprint" of a given sample. Different authors have extensively reviewed the advantages and limitations of Raman spectroscopy for molecular diagnosis and biological tissues characterization, such as cartilage (Eberhardt et al., 2015, Butler et al., 2016).

One of the advantages of RS is the possibility to irradiate *in vitro* or *in vivo* human samples without damaging the tissues. After irradiation, Raman spectra acquisition are usually displayed at different intensities with corresponding wavelength shifts (cm^{-1}) specific to vibrations of molecular bonds (Aguiar et al., 2015) measured in arbitrary units. Raw data will be typically pre-processed in order to avoid unwanted baseline interference and processing may involve peak normalization; thereafter, data can be classified according to unsupervised or supervised statistical methods, allowing sample classification (biomarkers) or diagnostic analysis, respectively, where the latest involves the use of additional information obtained from gold

standards, such as histopathology or radiography scores. Moreover cross-validation against biochemical tools is frequently included (Eberhardt et al., 2015).

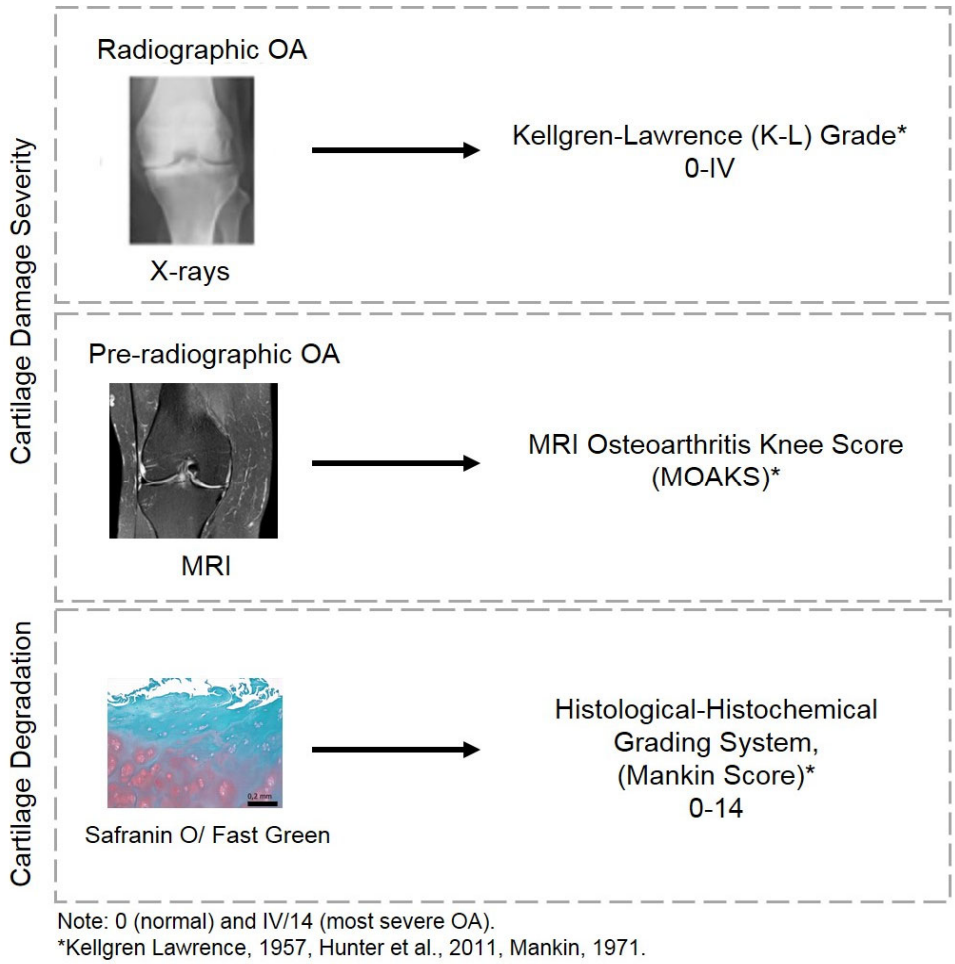

FIGURE 5.1. Commonly used semi-quantitative techniques for cartilage damage severity and degradation assessment.

Due to alternative processing pathways, data is not always suitable for direct comparison. Although quantitative comparison of results obtained from different studies may not possible, mean Raman spectra and 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 the spectral quality and detection accuracy (Querido et al., 2017).

Nonetheless, RS application in cartilage severity, degradation and repair assessment offers great potential as it can be performed *in situ* providing chemical and compositional high-quality data

without labeling, together with the fact this technique is minimally invasive, affordable and fast.

Raman spectroscopy application in OA diagnosis

Human cartilage molecular fingerprint by RS was first described by Esmonde-White (Esmonde-White et al., 2011a). Ever since, cartilage composition through spectral assignments from other joint tissues, such as tibial plateau, femoral condyle or femoral head, during OA, have been reported (Kumar et al., 2015, Takahashi et al., 2014, Gamsjaeger et al., 2014, Casal Beiroa et al., 2018a). As cartilage articular tissue is mainly constituted by collagens – with prevalent type-II (Col-II) – and glycosaminoglycans (GAGs) – being chondroitin sulfate (CS) the predominant sulfated GAG –, key alterations detected by RS involve vibrational and rotational shifts in these spectra components that can be associated with specific molecular events that occur during OA progression (Kumar et al., 2015).

Main changes described in the fingerprint range spectra (800-1800 cm^{-1}) have been recently reviewed and, as aforementioned, involve members of the collagen family, the proline/hydroxyproline distribution (doublet at 856-880 cm^{-1}) and amide III vibration (doublet at 1245-1270 cm^{-1}), associated to the secondary structure of proteins and their relative distribution; GAGs, the pyranose ring (band at 1039-52 cm^{-1}) and as representative of proteoglycans (PG) the peaks at ~1063 cm^{-1} and 1375 cm^{-1}; lipids, the 1441 cm^{-1} displacement; and a mineral phase in overdamaged tissues, attributed to hydroxyapatite (960 cm^{-1}), have been summarized in Fig. 5.2 (Casal-Beiroa et al., 2019b).

Moreover, different ratios for cartilage tissue degradation, such as cartilage-to-bone ratio (1063/960 cm^{-1}), mineralization ratio (960/920 cm^{-1}) and carbonate substitution ratio (1071/960 cm^{-1}), could also have predictive potential, as alterations were observed already in early stages of the disease (Hosu et al., 2019).

RS analysis, depending on the desired output, can be used both as exploratory analysis for pattern finding and biomarker extraction, and diagnostic analysis for spectral classification (Butler et al., 2016). In the case of OA some examples can be given, for example, Takahashi et al., were able to correlate microstructural information regarding amide III with surface morphological classifications based on Collins pathological scale, used for grading cartilage and bone changes (Takahashi et al., 2014).

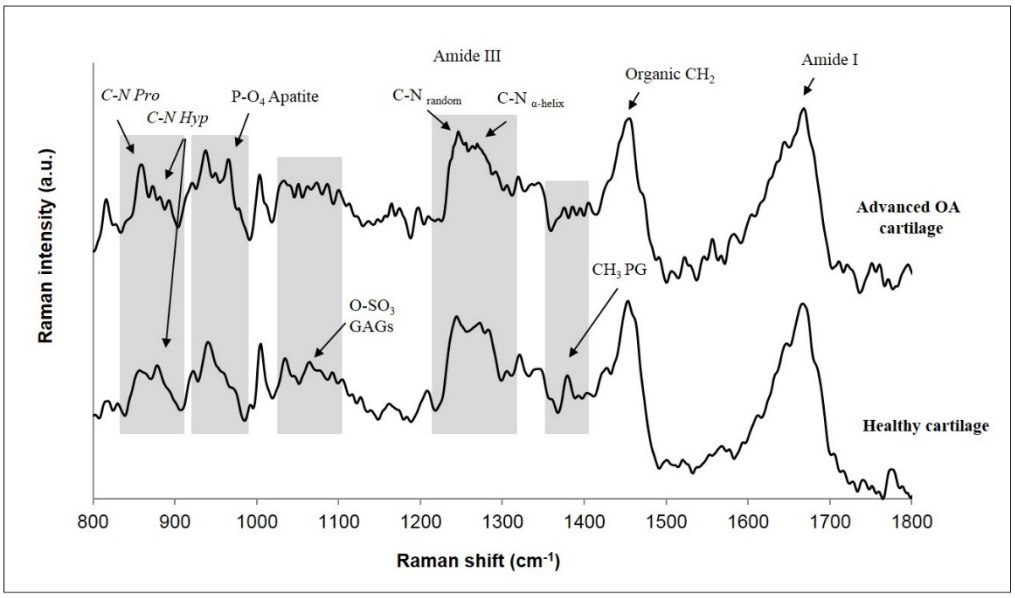

FIGURE 5.2. Human cartilage molecular fingerprint obtained from a healthy donor and an advanced OA donor. The analysis was performed *ex vivo* at a 1064 nm laser excitation and pre-processed with MagicPlot software for baseline correction

Later, multivariate principal component analysis (PCA) unsupervised approach was used for analyzing cartilage with different grades of OA (according to the International Cartilage Repair Society – ICRS – system), supporting previous findings as well as other alterations in PG specific band and an 85% efficiency in predicting the ICRS grade (Kumar et al., 2015).

Moreover, and beyond RS diagnostic potential for pathological articular cartilage damage during OA it may also be applied to other rheumatologic diseases (Hosu et al., 2019) or rare diseases affecting cartilaginous tissues (Taylor et al., 2019).

Raman spectroscopy pre-clinical applications: regenerative medicine

Beyond its potential for OA diagnosis and cartilage degradation monitoring, Raman spectroscopy could also be applied for the evaluation of other remodeling processes such as the ones that occur during cartilage repair and regeneration events. Thus, its usefulness could be extended to monitor clinical outcomes for specific interventions such as the ones addressed to treat pre-OA, focal lesions, or even to monitor *in vitro* expansion and pre-chondrogenic differentiation of either cells-only or cell-biomaterial constructs before implantation (Table 5.1) (Ember et al., 2017, von Erlach et al., 2015).

Jones et al. (Jones et al., 2005) were the first to apply Raman spectroscopy to monitor chondrocytes cellular response in macroporous bioactive glass foam scaffolds in real time. The

main bands found (amide I and III) correspond with allocations of extracellular matrix components and their intensity increased with culture time, being suggested as a measure of cartilage formation. Although no differences were established regarding the type of collagen synthesized, complementary studies on isolated collagens reported a displacement in amide I band maximum from ~1670 cm^{-1} (Col-II) to ~1655 cm^{-1} (Col-I) that could be used for validating Col-II synthesis during chondrogenesis (Dehring et al., 2006).

Monitoring during chondrogenic differentiation of other major components like proteoglycans, was studied using Raman microspectroscopy in cell cultures of both human and porcine chondrocytes, showing that this technique can differentiate tissue layers based on their composition, detecting an increase in 1064 cm^{-1} band (sulfated GAGs content) from superficial to middle and deep zones. Authors also found differences in bands at 817 and 921 cm^{-1} (proline vibrations) presenting higher intensities in the superficial zone. Chondrogenesis was further explored by other authors to establish neoformed cartilage reliability in comparison to native tissue (Bergholt et al., 2017a). Lately, other parameters like variation in collagen secondary structure, fibril orientation and relative gradients of components among tissue structure, have been described by Raman mapping and imaging (Albro et al., 2018, Bergholt et al., 2016b).

In order to allow effective real-time monitoring and encourage translation potential of RS based techniques to cell monitoring, Moura et al. demonstrated the non-invasiveness of live-imaging techniques in a dynamic follow-up of neo-formed tissue, by establishing a correlation between the lack of "morphological damage" with "molecular damage" and "growth impairment" in the chondrogenesis of fetal femur-derived skeletal cells, in a three-dimensional pellet culture system (Moura et al., 2018).

Other authors have focused on Raman application together with PCA to monitor chondrocytes' proliferation, in monolayer conditions, in real time. This study suggested the relevance of tracking bands at 1065, 1079 and 1300 cm^{-1} (sulfated GAGs and lipids) and their association with des-differentiation processes, indicating a decrease in PG and lipid content with culture time (Pudlas et al., 2013).

Moreover, a recent work has also shown the potential of RS as a sensitive and reproducible tool to routinely perform characterization of mesenchymal stromal cells (MSC)-derived extracellular vesicles (EVs). In this study, Raman spectra allowed the automatic distinction with a 93.7% accuracy among vesicles from three cytotypes (bone marrow-, adipose tissue-derived

MSCs and dermal fibroblasts) which can accelerate the translation of EVs clinical application as one of the next generation cell-free therapies (Gualerzi et al., 2017) for cartilage repair and osteoarthritis (Miyaki et al., 2018, Malda et al., 2016).

TABLE 5.1. Raman spectroscopy applications in cartilage development.

Cell type or tissue	Culture System	Main findings	References
Chondrocytes	3D scaffold	RS allows non-invasive motorization of collagen-like formation in real time	Jones et al., 2005
Bovine articular cartilage and chondrocytes	Agarose	RS imaging provides highly accurate and spatial resolution semi-quantitative data of ECM components' distribution in native and tissue engineered cartilage	Albro et al., 2018
Bovine articular cartilage and chondrocytes	Agarose	Fiber-optic RS allows non-destructive quantitative biochemical analysis of the ECM in live cell constructs	Bergholt et al., 2017a
Bovine articular cartilage and chondrocytes	Agarose	RS imaging identifies six differentiated zones in articular cartilage and detects changes in ECM components distribution over time	Bergholt et al., 2016b
Human fetal femur cartilage	-	Definition of zonal fetal femur cartilage molecular fingerprint. Dendrograms and PCA usefulness for tissue differentiation analysis by Raman microspectroscopy	Kunstar et al., 2012
Human fetal femur-derived skeletal cells	Monolayer or suspension	Multimodal microscopy system allows monitoring chondrogenic differentiation of fetal cells and elucidates temporal changes in cartilage development	Moura et al., 2018
Porcine/human chondrocytes, SW-1353 cell line and human cartilage	Monolayer	Raman microspectroscopy monitoring shows phenotypic changes of PG-related ECM components in chondrocytes after prolonged *in vitro* culture	Pudlas et al., 2013
Human MSCs derived-EVs and dermal fibroblasts	Suspension	RS distinguishes EVs derived from different cytotypes and discriminates main EVs' constituents in solution	Gualerzi et al., 2017
Commercial human MSCs (PromoCell)	Micropattern arrays	RS mapping combined with micro-engineering stem cell platform allows obtaining quantitative data from intracellular molecules such as proteins, lipids and other metabolites within cells	von Erlach et al., 2015

Raman spectroscopy can thus be presented as a label-free non-destructive alternative to conventional analytical methods that require previous sample preparation and/or tissue homogenization (Esmonde-White, 2014b).

Final Remarks

Raman spectroscopy is a non-invasive, label-free, highly sensitive technique for the detection and assessment of cartilage chemical and molecular composition with clinical translation potential for OA early diagnosis. Future steps include further standardization in processing methodologies, defining and validating optical biomarkers and fine-tuning laser equipment for clinical implementation. Furthermore, RS has also the potential to monitor *in vitro* cell expansion and differentiation processes prior to cell or cell-biomaterial implantation as well as an *in situ* follow up.

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References

Aguiar H., Rodríguez-Domínguez M., Coello B., Caeiro-Rey J. R., Stefanov S., Rodríguez-Valencia C., López E., López-Álvarez M., Chiussi S., Serra J., González P. (2015) Espectroscopia Raman para el análisis de tejido vivo y biocerámicas de origen biológico y sintético. In: Biomateriales. Diseño, producción y caracterización (ed.), Red Gallega de Biomateriales, Vigo, Spain, pp. 33-52.

Albro M. B., Bergholt M. S., St-Pierre J. P., et al. (2018) Raman spectroscopic imaging for quantification of depth-dependent and local heterogeneities in native and engineered cartilage. NPJ Regen Med, 3(1): 3.

Altman R., Asch E., Bloch D., et al. (1986) Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis Rheum, 29(8): 1039-1049.

Bergholt M. S., Albro M. B., Stevens M .M (2017) Online quantitative monitoring of live cell engineered cartilage growth using diffuse fiber-optic Raman spectroscopy. Biomaterials, 140: 128-137.

Bergholt M.S., St-Pierre J.P., Offeddu G.S., et al. (2016) Raman Spectroscopy Reveals New Insights into the Zonal Organization of Native and Tissue-Engineered Articular Cartilage. ACS Cent Sci, 2(12): 885-895.

Blanco F.J. (2014) Osteoarthritis: Something is moving. Reumatol Clinica, 10(1): 4-5.

Butler H.J., Ashton L., Bird B., Cinque G., Curtis K., Dorney J., Esmonde-White K., Fullwood N.J., Gardner B., Martin-Hirsch P.L., Walsh M.J., McAinsh M.R., Stone N., Martin F.L. (2016) Using Raman spectroscopy to characterize biological materials. Nature Protocols, 11(4): 664-87.

Casal Beiroa P., Burguera E.F., Hermida-Gómez T., Goyanes N., Oreiro Villar N., Blanco F.J., González P., Magalhães J. (2018) Optical biomarkers for the early diagnosis of osteoarthritis. Osteoarthr Cartilage, 26(1): S191.

Casal-Beiroa P., Balboa-Barreiro V., Goyanes N., Filgueira P., González P., Pertega-Díaz S., Blanco F.J., Magalhães J. (2019) Correlations between cartilage molecular composition by Raman spectroscopy and Mankin score: impact of inter- and intra-variability. Ann Rheum Dis, 78(Suppl 2): 947.2-948.

Dehring K.A., Crane N.J., Smukler A.R., McHugh J.B., Roessler B.J., Morris M.D. (2006) Identifying chemical changes in subchondral bone taken from murine knee joints using raman spectroscopy. Appl Spectrosc, 60(10): 1134-1141.

Deveza L.A., Melo L., Yamato T.P., Mills K., Ravi V., Hunter D.J. (2017) Knee osteoarthritis phenotypes and their relevance for outcomes: a systematic review. Osteoarthr Cartilage, 25(12): 1926-1941.

Eberhardt K., Stiebing C., Matthäus C., Schmitt M., Popp J. (2015) Advantages and limitations of Raman spectroscopy for molecular diagnostics: an update. Expert Rev Mol Diagn, 15(6): 773-87.

Ember K.J.I., Hoeve M.A., McAughtrie S.L., Bergholt M.S., Dwyer B.J., Stevens M.M., Faulds

K., Forbes S.J., Campbell C.J. (2017) Raman spectroscopy and regenerative medicine: a review. NPJ Regen Med, 2: 12.

Esmonde-White K.A., Esmonde-White F.W.L., Morris M.D., Roessler B.J. (2011) Fiber-optic Raman spectroscopy of joint tissues. Analyst, 136(8): 1675-1685.

Esmonde-White K.A. (2014) Raman spectroscopy of soft musculoskeletal tissues. Appl Spectrosc, 68(11): 1203-1218.

Gamsjaeger S., Klaushofer K., Paschalis E.P. (2014) Raman analysis of proteoglycans simultaneously in bone and cartilage. J Raman Spectrosc, 45(9): 794-800.

Gualerzi A., Niada S., Giannasi C., Picciolini S., Morasso C., Vanna R., Rossella V., Masserini M., Bedoni M., Ciceri F., Bernardo M.E., Brini A.T., Gramatica F. (2017) Raman spectroscopy uncovers biochemical tissue-related features of extracellular vesicles from mesenchymal stromal cells. Sci Rep, 7(1): 9820.

Hosu C.D., Moisoiu V., Stefancu A., Antonescu E., Leopold L.F., Leopold N., Fodor D. (2019) Raman spectroscopy applications in rheumatology. Lasers Med Sci, 34(4): 827-834.

Hunter D.J., Guermazi A., Lo G.H., Grainger A.J., Conaghan P.G., Boudreau R.M., Roemer F.W. (2011) Evolution of semiquantitative whole joint assessment of knee OA: MOAKS (MRI Osteoarthritis Knee Score). Osteoarthr Cartilage, 19(8): 990–1002.

Jones J.R., Vats A., Notingher I., et al. (2005) In Situ Monitoring of Chondrocyte Response to Bioactive Scaffolds Using Raman Spectroscopy. Key Eng Mater, 284-286: 623-626.

Karsdal M.A., Michaelis M., Ladel C., et al. (2016) Disease-modifying treatments for osteoarthritis (DMOADs) of the knee and hip: lessons learned from failures and opportunities for the future. Osteoarthr Cartilage, 24(12): 2013-2021.

Kellgren J.H., and Lawrence J.S. (1957) Radiological assessment of osteo-arthrosis. Ann Rheum Dis, 16(4): 494-502.

Kumar R., Grønhaug K.M., Afseth N.K., Isaksen V., de Lange Davies C., Drogset J.O., Lilledahl M.B. (2015) Optical investigation of osteoarthritic human cartilage (ICRS grade) by confocal Raman spectroscopy: a pilot study. Anal Bioanal Chem, 407(26): 8067-77.

Mankin H.J., Dorfman H., Lippiello L., Zarins A. (1971) Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J Bone Joint Surg Am, 53(3): 523-37.

Malda J., Boere J., van de Lest C.H., van Weeren P., Wauben M.H. (2016) Extracellular vesicles — new tool for joint repair and regeneration. Nat Rev Rheumatol, 12(4): 243-9.

Miyaki S. and Lotz M.K. (2018) Extracellular vesicles in cartilage homeostasis and osteoarthritis. Curr Opin Rheumatol, 30(1): 129-135.

Moura C.C., Lanham, S.A., Monfort, T., Bourdakos K. N., Tare, R. S., Oreffo, R. O. C., Mahajan, S. (2018) Quantitative temporal interrogation in 3D of bioengineered human cartilage using multimodal label-free imagig. Integr Biol, 10(10): 635-645.

Musumeci G., Aiello F.C., Szychlinska M.A., Di Rosa M., Castrogiovanni P., Mobasheri A. (2015) Osteoarthritis in the XXIst century: risk factors and behaviours that influence disease onset and progression. Int J Mol Sci, 16(3): 6093-6112.

Pauli C., Whiteside R., Heras F.L., Nesic D., Koziol J., Grogan S.P., Matyas J., Pritzker K.P., D'Lima D.D., Lotz M.K. (2012) Comparison of cartilage histopathology assessment systems on human knee joints at all stages of osteoarthritis development. Osteoarthr Cartilage, 20(6): 476-85.

Pascual Garrido C., Hakimiyan A.A., Rappoport L., Oegema T.R., Wimmer M.A., Chubinskaya S. (2009) Anti-apoptotic treatments prevent cartilage degradation after acute trauma to human ankle cartilage. Osteoarthr Cartilage, 17(9): 1244-51.

Pearson R.G., Kurien T., Shu K.S., Scammell B.E. (2011) Histopathology grading systems for characterization of human knee osteoarthritis--reproducibility, variability, reliability, correlation, and validity. Osteoarthr Cartilage, 19(3): 324-31.

Pudlas M., Brauchle E., Klein T.J., Hutmacher D.W., Schenke-Layland K. (2013) Non-invasive identification of proteoglycans and chondrocyte differentiation state by Raman microspectroscopy. J Biophotonics, 6(2): 205-211.

Querido W., Falcon J.M., Kandel S., Pleshko N. (2017) Vibrational spectroscopy and imaging: applications for tissue engineering. Analyst, 142(21): 4005-4017.

Takahashi Y., Sugano N., Takao M., Sakai T., Nishii T., Pezzotti G. (2014) Raman spectroscopy investigation of load-assisted microstructural alterations in human knee cartilage: Preliminary study into diagnostic potential for osteoarthritis. J Mech Behav Biomed Mater, 31: 77-85.

Taylor A.M., Jenks D.D., Kammath V.D., et al. (2019) Raman Spectroscopy identifies differences in ochronotic and non-ochronotic cartilage; a potential novel technique for monitoring ochronosis. Osteoarthr Cartilage, 27(8): 1244-1251.

van der Kraan P.M., Berenbaum F., Blanco F.J., et al. (2016) Translation of clinical problems in osteoarthritis into pathophysiological research goals. RMD Open, 26;2(1): e000224.

von Erlach T.C., Hedegaard M.A., Stevens M.M. (2015) High resolution Raman spectroscopy mapping of stem cell micropatterns. Analyst, 140(6): 1798-803.