Osteoarthritis and Cartilage

Review

Molecular and structural imaging in surgically induced murine osteoarthritis

N.H. Lim † *, C. Wen ‡*, T.L. Vincent ‡

† Centre for Osteoarthritis Pathogenesis Versus Arthritis, Kennedy Institute of Rheumatology, University of Oxford, UK
‡ Department of Biomedical Engineering, Hong Kong Polytechnic University, Hong Kong

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Summary

Preclinical imaging in osteoarthritis is a rapidly growing area with three principal objectives: to provide rapid, sensitive tools to monitor the course of experimental OA longitudinally; to describe the temporal relationship between tissue-specific pathologies over the course of disease; and to use molecular probes to measure disease activity in vivo. Research in this area can be broadly divided into those techniques that monitor structural changes in tissues (microCT, microMRI, ultrasound) and those that detect molecular disease activity (positron emission tomography (PET), optical and optoacoustic imaging). The former techniques have largely evolved from experience in human joint imaging and have been refined for small animal use. Some of the latter tools, such as optical imaging, have been developed in preclinical models and may have translational benefit in the future for patient stratification and for monitoring disease progression and response to treatment. In this narrative review we describe these methodologies and discuss the benefits to animal research, understanding OA pathogenesis, and in the development of human biomarkers.

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Introduction

Our understanding of osteoarthritis (OA) pathogenesis has greatly increased since the advent of surgical models of OA in genetically modified mice1. An exponential increase in in vivo studies since this time has identified major molecular players in disease as well as excluding others2-7. Whilst it is difficult to prove the clinical utility of murine models when there are so few successes in clinical practice, where they do exist, the results accord well. For instance, the lack of efficacy demonstrated by anti-cytokine therapy and the identification of nerve growth factor (NGF) as a target for OA pain holds true for both human and murine disease7-10.

Pathological changes in mouse joints following surgical destabilisation are largely determined by histological assessment at multiple levels within the joint and mirror human disease well; demonstrating progressive cartilage degradation, osteophyte formation, modest synovial hypertrophy and late onset spontaneous pain behaviour. Although in vivo models were principally developed and validated to assess cartilage degradation as the main outcome measure, increasingly semi-quantitative assessments of bone and synovium are also being included in preclinical studies. The relative importance of each of these pathological features to symptomatic disease and cartilage loss is hotly debated in clinical and pre-clinical arenas. The ability to interrogate molecules in specific tissues of the joint by creating conditional knockout mice is likely to help elucidate these issues.

Animal imaging, both in vivo (live) and ex vivo (dead), offers adjunctive information that could be transformative in terms of screening genetically modified animals, understanding pathogenesis, and creating tools that could be useful for human disease monitoring. Specifically these include: (i) the development of rapid quantitative measures that circumvent the need for laborious histological processing and scoring in preclinical models; (ii) the development of prospective measures that could be used in live animals to follow the course of disease in an individual animal over time. This would have a significant impact on reduction of animals in research in line with ARRIVE guidelines; (iii) the ability to use

* Address correspondence and reprint requests to: N.H. Lim, Centre for Osteoarthritis Pathogenesis Versus Arthritis, Kennedy Institute of Rheumatology, University of Oxford, UK.
E-mail address: han.lim@kennedy.ox.ac.uk (N.H. Lim).

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Computer tomography (CT)

Computer tomography (CT) is one of the most widely used in vivo and ex vivo imaging modalities in orthopaedic research. The mineral calcium in bone absorbs the X-rays and by rotating either the specimen or the X-ray source and detector, 3D data of the bone is reconstructed from multiple projections at multiple angles around the specimen. With technological advancements in detector resolving power and computer processing power, high resolution ex vivo nanoCT scans of down to 50–600 nm per voxel are possible, allowing clear visualisation of minute features like osteocyte lacunae and vascular canals within a trabecula. In vivo microCT scans are of lower resolution, in the 4.5–50 μm per voxel range, as it needs to balance motion artefact and the radiation dosage per scan. Oversampling with image binning overcomes motion artefact caused by heartbeats and respiration, but this increases the radiation exposure. In vivo CT allows the same animal to be followed throughout the study. Apart from reducing the number of animals required, this provides greater insight into the temporal development of structural disease, such as the increase in new bone (osteophytes) after surgical destabilisation of the medial meniscus (DMM) (Fig. 1).

Parameters that are typically assessed from CT scans in experimental OA include subchondral bone sclerosis and osteophyte size (Fig. 2). Osteophytes are defined as new bone formation and typically occur on the medial side of the joint following meniscal transection. Osteophytes are sometimes observed as small hook-shaped projections at the edge of the joint in CT scans, but they can also be inferred by an increase in volume of the epiphysis. Validation of osteophytes is performed by histology, in which new bone boundaries are clearly visible, and automated methods can be applied to speed up analysis. Such quantitative volume assessments by microCT appear to map well to the development of the osteophyte by histology. Both subchondral bone thickening and osteophyte formation occur progressively after joint destabilisation. These are evident within the first couple of weeks of surgery by histology and epiphyseal volume measurements. MicroCT has also been used to quantify osteophyte number, to describe bony deformity in genetically modified joints and abnormal ossification of soft tissues of the joint. Bone density measurements within the epiphysis are also measured although whether such features inform pathological processes in OA is unclear.

Cartilage is not visible by conventional CT unless a contrast agent is used. As with human contrast enhanced imaging, agents can either be anionic, thereby being excluded from GAG-rich matrix, or cationic, thereby being enhanced within cartilage. Both anionic and cationic contrast agents have been tested in rodent models of OA. The correlation between cartilage damage assessment using this type of approach compared with histological scoring is excellent (adapted in Fig. 3). The different classes of disease activity probes that provide real time information on cellular processes associated with disease.

In addition to the benefits to the preclinical OA community, ultimately such imaging outcomes have clinical utility as biomarkers. The lack of sensitive biomarkers for OA greatly hampers progress in translation. The only widely accepted biomarker for OA is the Kellgren and Lawrence radiographic score, a composite of joint space narrowing and osteophyte formation, measured by plain X-ray. Joint space narrowing as a biomarker is insensitive, with only around 30% of individuals recruited to OA trials showing plain X-ray. Joint space narrowing as a biomarker is insensitive, with only around 30% of individuals recruited to OA trials showing

| Table I | Comparison of pre-clinical imaging modalities |

| Imaging type | μMRI | μCT | Ultrasound | PET | SPECT | Optical | Opto-acoustic |
|-------------|-----|-----|-----------|-----|-------|---------|-------------|
| Resolution  | Structural | Structural | Structural | Molecular | Molecular | Molecular | Molecular |
| Contrast agent | Gadolinium | Iodine | Microbubbles | 18F | 99mTc | Fluorophore | Light-absorber |
| Typical amount of contrast required | mg | mg | ng | ng | ng | ng-μg | ng-μg |
| Acquisition Time | > 10 min to hours | < 10 Mins | < secs | > 10 min | > 10 min | Secs | < sec |
**Fig. 1**
Prospective *in vivo* CT scans following changes within a joint after DMM or sham surgery (10 μm/pixel resolution). DMM surgery induces extrusion of the meniscus which calcifies in week 4 (red arrow). Ossification also occurs in the destabilised joint leading to formation of an osteophyte (week 8) (red arrow) (Lim, unpublished data).

**Fig. 2**
MicroCT changes in subchondral bone over time in spontaneous OA in the Hartley guinea pig. Note subchondral bone plate thickening and increased cystic lesion in underlying trabecular bone (Wen, unpublished data). Solid arrow — bone sclerosis; hashed arrow — cystic bone lesion.

**Fig. 3**
Quantitative cartilage volume assessment using *ex vivo* contrast enhanced microCT following DMM (A) *Ex vivo* tibia were stained with phosphotungstic acid and imaged by microCT and histology at 4 weeks post DMM (B) Histopathology scores of the medial condyles at 4 and 12 weeks post DMM (C) A good correlation is evident between the two cartilage thickness measurements using the two methods. Adapted from 14, with permission.
contrast agents penetrate cartilage at different speeds and their in vivo utility can be broadened by adjusting the time of image acquisition after delivery, route of contrast agent delivery (intra-articular vs systemic) and dose (reviewed in 25).

Contrast-enhanced nanoCT provides good spatial and contrast resolution equivalent to histological staining, and can provide fast and quantitative data on cartilage structure 13. However, nano-CT is resolution equivalent to histological staining, and can provide fast and quantitative data on cartilage structure 13. However, nano-CT is not yet suitable for live imaging due to extreme sensitivity to motion artefact. Synchrotron-radiation CT (SRCT), which generates high energy X-rays of narrower wavelength ranges, improves the resolution of conventional CT greatly. Intercortical pores in ex vivo murine bones are easily observed using SRCT 26 and bones imaged by SRCT in vivo have better bone boundaries when imaged at a dose that is comparable to usual microCT, due to phase enhancement 27. While conventional CT is poor at differentiating non-calciﬁed tissues, Marenzana et al. demonstrated the use of SRCT for imaging mouse articular cartilage without the need for contrast agents 28.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) utilises a strong electromagnetic to generate a changing magnetic field at a frequency close to the natural frequency of hydrogen atoms. This allows hydrated tissues to be visualised well. Theoretically, MR imaging techniques allow optimal detection of several different joint tissues; from articular cartilage, meniscus, subchondral bone to synovium. Murine OA has been examined using a 9.4T preclinical micro-MRI scanner with an isotropic resolution of 0.068 mm/pixel for T2 images and 0.12 mm/pixel for T1 images 29. However, the thickness of murine cartilage (80–100 μm) is close to the resolution limit of micro-MRI. Therefore, T2-weighted images are limited to detecting subchondral bone oedema-like changes and T1-weighted images to assessing perfusion abnormalities in subchondral bone after injection of contrast agent (Fig. 4). Subchondral bone edema and perfusion abnormalities were associated with increased angiogenesis which temporally preceded and spatially localised with bone and cartilage lesions 30. Longitudinal micro-MRI imaging in surgically induced OA in rats showed that subchondral edema developed subsequently into cystic lesions 30. Similar observations were also documented in spontaneous OA in the Dunkin Hartley guinea pig 31.

The T2 relaxation time has also been derived to indicate structural integrity of articular cartilage in larger rodent models by probing free water proton movement inside the collagen-proteoglycan matrix. Indeed, T2 mapping has been used to detect proteoglycan loss and ECM alterations in rat patellar cartilage post hyaluronidase digestion 32, and in spontaneous OA in the Dunkin Hartley guinea pig in vivo 33. In a recent study by Ali et al., pathological changes in the joint were assessed over time following rat meniscectomy. This study describes an initial swelling of the articular cartilage, subchondral bone remodelling, then proteoglycan depletion and cartilage erosion over 8 weeks 34. Strategies that improve the contrast and speciﬁcity in MRI, such as by sodium MRI or gadolinium (Gd), have been used in larger animals and may eventually have use in rodents 33,35. Gd-containing cartilage targeting contrast agents, based on type II collagen binding, have been used to image cartilage in rats 33.

Ultrasonography

Ultrasonography has been used in clinical assessment of hand, hip and knee OA from grading synovitis, to measuring cartilage thickness, damage to the meniscus and assessing the cartilage–bone interface. In preclinical models, B-mode images have been used to assess synovitis in inﬂammatory arthritis 36. Newer ultra-high frequency linear array transducers, such as MS700 with central frequency 30–70 MHz, have been developed with multi-focus capability to enhance tissue margin visualisation and provide improved morphological information such as synovial or meniscal swelling post injury 37. Power Doppler can be applied to monitor joint blood ﬂow over time, for example after destabilization of the meniscus where changes in vascularity are evident (Fig. 5). Doppler imaging is reliant on moving objects in blood vessels, e.g., erythrocytes, and has a resolution down to 30 μm at an ultrasound probe frequency of 70 MHz. Cardiac pumping and respiratory rate of experimental mice under general anesthesia are major factors affecting power Doppler signals. Cardiac and respiratory gating can overcome this technical problem. B-mode ultrasound and Power Doppler are qualitative and often operator-dependent. Quantitative 3D Power Doppler ultrasonography has, to some extent, overcome operator-dependent observational bias and has been used to study posttraumatic murine osteoarthritis in a...
longitudinal follow-up study\textsuperscript{39,40}. In this study, the authors determined joint space and blood flow volume of the joint. Interestingly, the change in blood flow volume determined by Power-Doppler was not apparent until 6-week post operation\textsuperscript{40}. Ultrasonography has also been used ex vivo to study the joint surface (also termed biomicroscopy)\textsuperscript{41}. Developing conjugated probes for arthroscopic use may provide future solutions\textsuperscript{42}. Tissue resolution and penetration of ultrasonography currently limits its in vivo use; there being a trade-off between frequency of the ultrasound wave and penetration depth. Specifically it is unable to penetrate bone. Radiofrequency analysis of sonic signals at the bone-cartilage interface might be useful to delineate this functional unit during OA progression\textsuperscript{43}.

**Molecular outcomes**

Molecular imaging techniques detect selective molecular activities which can be monitored over the course of disease. Their value lies in the ability to explore pathogenetic mechanisms and validate therapeutic targets in real-time.

**Positron emission tomography (PET)-CT**

Positron Emission Tomography (PET) detects gamma rays emitted indirectly by positron-emitting radionuclide tracers. $^{18}$F-Fludeoxyglucose (FDG), which is the most commonly used positron emission tomography (PET) tracer, is taken up at higher rates at regions of heightened metabolic activity, e.g., cancer metastases. When used in combination with CT (PET-CT) skeletal localisation can be elucidated. Umemoto and colleagues examined rat ACLT-induced OA using $^{18}$F-fluoride PET and observed higher uptake of $^{18}$F in the operated knee starting 2 weeks post operation, particularly within the medial compartment\textsuperscript{44}. Macrophage activity following induction of OA by mono-iodoacetate (MIA) was followed by PET using a $^{64}$Cu-peptide targeted to the formyl peptide receptor 1, which is up-regulated upon macrophage activation\textsuperscript{45}.

**SPECT**

Single photon emission computer tomography (SPECT) is generated by use of a gamma-emitting radioisotope e.g., $^{99m}$Technetium-methylene diphosphonate radiotracer\textsuperscript{46}. Activated macrophages were identified in the rat groove model of OA following SPECT imaging using a new DOTA-folate radio-conjugate\textsuperscript{47}. A radio-tracer that was excluded from bone and synovium was used to image cartilage by SPECT\textsuperscript{48}.

Improvements have been made to the pinhole design and camera design to improve resolution, magnification and detection efficiency\textsuperscript{49}. The newest generation $\mu$SPECT system can resolve details down to 0.25 mm\textsuperscript{50}. Furthermore, Hybrid SPECT/CT and SPECT/MRI systems have been designed and built for preclinical purposes to allow researchers to acquire functional images and structural images simultaneously, thereby understanding the correlations between metabolic activities and structural alterations.

**Optical Imaging**

Optical imaging detects light emitted in the visible to near infrared wavelengths. This light may either be from a bioluminescent source, or the longer emission wavelength of a fluorophore excited at a shorter wavelength of light (fluorescence). This makes it more accessible than the traditional PET/SPECT molecular imaging, as no radioactive material needs to be handled. The main drawbacks of optical imaging stem from the natural tendency of the tissues of the body to absorb and scatter light, leading to low spatial resolution and an imaging depth of about 1–1.5 cm.

**Optical imaging - bioluminescence**

Gene expression changes can be studied in vivo using reporter mouse lines containing a luciferase gene under the control of a promoter of interest. Whole body optical imaging systems are used to capture the light emitted following injection of the luciferin substrate. The expression of aggrecan\textsuperscript{52} and NF$\kappa$B\textsuperscript{53} post-DMM surgery and NF$\kappa$B post-MIA injection\textsuperscript{54} have been reported using the corresponding reporter mice\textsuperscript{55}. The expression of NF$\kappa$B demonstrated a correlation with pain\textsuperscript{56} and a phase of increased NF$\kappa$B expression immediately following DMM or sham surgery but lasting longer in the DMM group\textsuperscript{57}.

Luciferase reporters can also be used in transplanted cell tracking experiments using the same optical imaging systems to demonstrate the presence of exogenous cells in repairing tissues of OA rats following ACLT\textsuperscript{55,56} and persistence of transplanted senescent auricular chondrocytes when injected into mouse joints to cause OA like disease\textsuperscript{57}.
Optical imaging — fluorescence

Whole body in vivo optical imaging systems can also track and quantify far red and near infrared fluorescence. Applications include far red expressing reporter genes or conjugation of the fluorophore to a variety of molecules including recombinant proteins, antibodies, peptides, nanoparticles and drugs. In our experience, the sensitivity of detection of far-red fluorescent protein when expressed by cells is about an order of magnitude less than luciferase.

The far-red probe Cy5.5 has been conjugated to an antibody selective for reactive-oxygen damaged type II collagen. When delivered to mice following DMM surgery evidence of oxidative damage could be detected as early as 4 weeks post op, preceding histological changes. Nanosomes encapsulating a near infrared dye and conjugated to an antibody that binds native type II collagen were retained by OA knees at a stage of modest superficial damage suggesting that this may be a sensitive marker of early disease.

Collagen hybridising peptides have been used to detect stretch induced damage to tendons through their binding to exposed collagen triple helices. Although not yet applied to in vivo models, these would potentially make interesting probes to test in OA. Fluorescence imaging of aggregan content in murine cartilage has been performed ex vivo using octaarginine-labelled with rhodamine, a methodology based on octaarginine’s positive charge.

Photoacoustic imaging

Photoacoustic (PA) imaging, also known as optoacoustic imaging, is a derivative of ultrasonography. Unlike typical ultrasonography, no acoustic transducer is involved. Instead, a non-ionising laser source sends light to the tissues. This light energy is absorbed by the tissue and converted into heat, and it is this heat that drives rapid thermoelastic expansion, which in turns causes ultrasonic acoustic signal to be emitted. Emerging PA imaging exhibits strengths of both optical and sonic imaging, enabling one to probe deeper into tissue than their optical counterparts do.
the optical absorption properties of endogenous haemoglobin in blood vessels relatively deep in the tissue while achieving the spatial resolution of ultrasound. PA imaging can be applied to in vivo non-invasive measurements of tissue angiogenesis and oxygenation levels without contrast agent. Compared with Power Doppler, PA imaging appears more sensitive for in vivo detection of intra-articular tissue damage and angiogenesis. Following DMM in mice, a reduction in oxygen saturation and increase in vascularity correlated with cartilage damage over time (Fig. 6). Changes in PA signal from the subchondral bone have been reported, albeit in a joint immobilisation model in rats. A cartilage contrast agent for PA imaging, utilising melanine nanoparticles encapsulated in poly-L-lysine, has shown good correlation with tissue glycosaminoglycan (GAG) content and is able to distinguish early from late OA.

Multispectral optoacoustic tomography (MSOT) is an advancement on PA and uses multiple wavelengths of light coupled with unmixing algorithms to excite both endogenous and exogenous light absorbing sources. MSOT, in combination with contrast, has been used successfully in inflammatory arthritis models and may have future application in OA imaging.

Protease-activated probes
The ability to monitor specific protease activity in vivo in real time has huge potential both in terms of understanding the chronology of matrix degradation in OA but also as a biomarker for selecting or predicting response to a particular treatment. The probes work by utilising a cleavable peptide sequence, which only fluoresces upon cleavage (Fig. 7). The selectivity of the probe depends on the selectivity of the sequence of the peptide substrate. The absence of sensitive assays that would allow degradative processes to be measured quantitatively in OA tissues means that these types of approaches are especially valuable, but not so easy to validate.

Commercial activity based probes exist for the matrix metalloproteases matrix metalloproteinases (MMPs) and Cathepsins, with the brand names MMPSense and Prosense. Activity of these probes has been described in collagenase-induced OA as well as surgical destabilisation and mechanical injury models of OA. Unsurprisingly, highest signals have been obtained with the more inflammatory models and early in the post-operative period. Cathepsin activity in particular appears to be more evident in inflammatory arthritis models such as collagen-induced arthritis rather than after DMM. The Sensitivity of MMPSense is improved when the probe is delivered intra-articularly following DMM. Prosense and MMPSense have also been used to monitor the efficacy of protease inhibition by the general protease inhibitor α2-macroglobulin and its more targeted variants. These commercial probes, particularly MMPSense, were developed for cancer monitoring, and are not selective for particular MMPs, so activity cannot be assigned to a particular MMP without additional verification.

**Fig. 7**
Schematic of protease specific probe activation. F-Star represents the fluorophore, Q-Hexagon the quencher and blue oval a chemical inhibitor moiety (A) A basic activity probe. In the intact probe, the fluorophore and quencher are in close proximity and any excitation of the fluorophore results in the transmission through fluorescence resonance energy transfer to the quencher. Upon cleavage, these separate and fluorescence is detectable (B) Activity probes attached to a polymer (e.g., MMPSense), without a chemical quencher. The high density of fluorophores results in self-quenching. Cleavage by protease releases some fluorophores and an increase in fluorescence (C) Activity probes with a hidden inhibitor moiety that will bind to the active site of the protease upon cleavage.
Probes with different specificities used in tandem may provide such validation. The different activation times of CatK680 compared with Prosense680 in the mechanical loading model suggests that Cathepsin K is activated early, whereas Cathepsin B, S, L or plasmin may be responsible for the later cleavage of Prosense68076. MMP13ap, which has moderately high selectivity for MMP-13 (also cleaves MMP-12), has been assessed after DMM in conjunction with MMP12ap (selective for MMP-12)72. As there was no regulation of MMP-12 observed with the MMP12ap probe, it is a reasonable assumption that the MMP13ap was indeed reporting on MMP-13 activity. Determining specificity may also be aided by parallel messenger ribonucleic acid (mRNA) analysis. For example, activation of MMPSense750 in the loading model may be due to MMP-3, as its mRNA was the upregulated, whereas MMP-9 and MMP-13 activity. Determining specificity may also be aided by parallel messenger ribonucleic acid (mRNA) analysis. For example, activation of MMPSense750 in the loading model may be due to MMP-3, as its mRNA was the upregulated, whereas MMP-9 and MMP-13 were unchanged75.

Newer probes with increased specificity have been developed and it is beginning to be possible to elucidate the in vivo protease cascade during disease. The peptide sequence of the activity-based probe may be designed to increase the selectivity for a particular family member over the others. A peptide sequence, identified from a phage display library screen using MMP-13, was modified and used in an anterior cruciate ligament tear (ACLT) and menisectomy rat model and showed increased cleavage 6 and 8 weeks post-surgery73. A second generation probe showed reduced cleavage by MMP-2 and MMP-9, but was still cleaved by MMP-774. Another method of obtaining selective peptide sequences is to reverse a selective peptide inhibitor into a substrate75, which was how MMP13ap was developed. The most recent reported MMP-13 probe (P-18) has used unnatural amino acids in order to achieve an almost 10-fold selectivity over the major confounding MMPs; MMP-2 and MMP-1275(Fig. 8). Coupled to a polyglutamic acid carrier (PGA-P-18), MMP-13 activity was detected 6 weeks post-DMM in mice and allowed the real-time readout of the efficacy of the MMP-13 inhibitor, A4727.

Development of probes for other proteases implicated in OA, such as the aggrecanases (ADAMTS-4 and ADAMTS-5), may provide earlier detection of OA and may allow stratification of targeted therapies.

**Conclusion**

Radiographic biomarkers in preclinical disease have the ability to help unravel pathogenic processes relevant to human disease and in combination with ex vivo molecular approaches, define the role of each tissue over the course of the disease. When measured prospectively, radiography has the ability to monitor disease progression in real time; reducing numbers of animals used and increasing the power of studies. Although contrast enhanced imaging of cartilage, either through microCT or photoacoustic imaging shows promise in vitro, these agents are not yet validated in vivo and the hope of finding a prospective imaging tool that will substitute for histological scoring of mouse joints is not currently within reach. Imaging probably holds more promise for those structural joint features that are less often reported; bone change, synovial vascularity and bone marrow oedema. Prospective imaging of bone remodelling after joint destabilisation is achievable by microCT scanning although caution should be taken not to overinterpret ossification of soft tissues, such as the meniscus, which occurs readily in mice as they age. Our experience is that epiphyseal volume change, the measurement of which can be automated, may be a more clinically relevant outcome measure76. Synovial hyper trophy and metabolic tissue status is not appreciated well by joint histology, so ultrasound technologies can add significantly to our understanding of the role of this tissue in OA. Limited resolution of microMRI continues to restrict its utility in non-inflammatory models of murine OA, although it is the only imaging tool currently able to visualise bone marrow oedema.

Newer molecular probes are being validated in pre-clinical models and these offer significant potential; from charting the natural history of specific protease activities over the course of disease to validating new therapeutic interventions. Other aspects of disease pathogenesis, such as molecular determinants of pain could be explored using PET/SPECT, although current resolution will likely preclude precise tissue localisation in vivo. Ultimately, molecular imaging has important clinical translational promise in the search for early diagnostic, therapeutic and prognostic biomarkers.

**Contributions**

TV and NHL conceived the review. NHL and CW collected data. All authors contributed to the writing of the manuscript and approved the final version.

**Conflict of interest**

TLV and CW have no conflicts of interest to declare. NHL has a pending patent on diiodotyrosine containing contrast agents for use in cartilage imaging (WO2018020262A).
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