QUANTIFICATION OF OSTEOCHONDRAL TISSUE MODIFICATIONS DURING OSTEOARTHRITIS USING MICRO-COMPUTED TOMOGRAPHY
SAKARI KARHULA

QUANTIFICATION OF OSTEOCHONDRAL TISSUE MODIFICATIONS DURING OSTEOARTHRITIS USING MICRO-COMPUTED TOMOGRAPHY

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium P117 (Aapistie 5B), on 16 November 2018, at 12 noon
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Abstract
Osteoarthritis (OA) is a heterogenic joint disease significantly affecting the quality of life of a patient, causing pain and disability. OA causes degenerative changes to the structure and composition of articular cartilage and subchondral bone. Currently, effective treatments for OA are limited, partly due to limitations in defining the imaging biomarkers of early OA. Improvement of imaging modalities in OA research and clinical setup is a requirement for quantitating early OA-related tissue features. In the clinical and preclinical setup, computed tomography (CT) enables imaging of bone and, using specific contrast agents, articular cartilage. The aim of this study is to create and validate novel micro-computed tomography (μCT) methods to quantify OA-related features and modifications in articular cartilage and subchondral bone.

Contrast-enhanced μCT methods for imaging the collagen (phosphotungstic acid (PTA) and phosphomolybdic acid (PMA)) and GAG (CA4+) content of the articular cartilage in vitro were validated against various reference methods measuring the biochemical composition of articular cartilage. To improve the μCT imaging of subchondral bone, grey-level co-occurrence matrix (GLCM) based analysis of sub-resolution features of subchondral bone was introduced. In addition, to test the translatability of the GLCM-based analysis to clinical resolution, sub-resolution features extracted from clinical cone-beam CT were validated against the subchondral bone morphometrics from the μCT.

PTA showed stronger association with the collagen content of the articular cartilage compared to PMA. PTA was also associated with collagen content even in degraded articular cartilage. CA4+ distribution was found to accumulate in chondrons and surrounding areas, suggesting that it is a prominent contrast agent for high-resolution μCT studies of chondrocytes. The GLCM-based analysis of subchondral bone provided information on cellular structure from μCT images and trabecular bone micro-structures from clinical CT images.

In conclusion, μCT imaging can provide quantitative information on the collagen content and chondrons of articular cartilage, as well as on osteocytes in subchondral bone. The methods presented here extend the tools for researchers to quantify osteochondral tissue modifications in OA. Furthermore, the developed image processing tools could be translatable to the clinical CT.

Keywords: articular cartilage, bone structure, cartilage composition, computed tomography, contrast agent, osteoarthritis, subchondral bone
Karhula, Sakari, Nivelrikon nivelerusto- ja luumuutosten määrittäminen mikrotietokonetomografialla. Oulun yliopiston tutkimuskoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Infotech Oulu Acta Univ. Oul. D 1492, 2018
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Tiivistelmä


Varjoaineavusteisia μTT in vitro menetelmiä ruston kollageenin (fosfoloframihappoa (PTA) ja fosfomolybdeenihapoa (PMA)) ja GAG (CA4+) jakauman määrälliseen tutkimukseen valitut uutisia on saattaa eri ruston biokemiallista koostumusta mittaavilla vertailumenetelmissä. Ruston- ja rustonalaisen luun kuvantamista kehitettiin soveltamalla harmaasävyje n tekstuurianalyysiä, jolla pyrittiin tunnistamaan kuva-alkioja suuremmiä luurakenteita. Ruston- ja rustonalaisen luun μTT-kuvien analyssein tulokset validoitettiin synkrotronisateliiteen perustuvan μTT:n avulla. Lisäksi tekstuurianalyysin sovelluvuutta testattiin klinisen resolution kartiokeilan TT-kuvilla. Kuvista analysoituja tekstuuriparametrejä verrattiin μTT:llä mitattuun todelliseen ruston- ja rustonalaisen luun rakenteeseen.

Vääristeisiin tuulet tuoretavat, että μTT on spesifimpi kollageenille testattuja varjoaineista ja sen sulla on verrannollinen kollageenijakaumaan jopa rappeutuneessa nivelrustossa. GAG-specifisen varjoaineen CA4+:n todettiin kerääntyvän myös kondroneihin, mikä viittaa siihen, että kyseinen varjoaine soveltuu potentiaalisesti rustosolujen korkean resolution μTT-tutkimuksiin. Ruston- ja rustonalaisen luun μTT-kuvista analysoituja tekstuuriparametreihin havaittiin olevan verranollisia osteosyyttien tilavuusfraktion kanssa.


Asiakirjat: luu, luuk, nivelrikko, nivelrusto, ruston koostumus, tietokonetomografia, varjoaine
“Enhance”

- Deckard (played by Harrison Ford) in *Blade Runner* (1982). Popularised by CSI and countless TV shows and movies with scenes of enhancing image resolution.

*To Linda and Ohto*
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Oulu, September 2018
Sakari Karhula
**Abbreviations**

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<tr>
<td>2D</td>
<td>two-dimensional</td>
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<td>3D</td>
<td>three-dimensional</td>
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<tr>
<td>ASM</td>
<td>angular second moment</td>
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<td>BV/TV</td>
<td>bone volume fraction</td>
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<tr>
<td>CBCT</td>
<td>cone-beam computed tomography</td>
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<tr>
<td>CEµCT</td>
<td>contrast-enhanced micro-computed tomography</td>
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<tr>
<td>CT</td>
<td>computed tomography</td>
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<td>FCD</td>
<td>fixed charge density</td>
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<tr>
<td>FD</td>
<td>fractal dimension</td>
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<td>FTIRI</td>
<td>Fourier transform infrared spectroscopy imaging</td>
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<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>GLCM</td>
<td>grey-level co-occurrence matrix</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>HU</td>
<td>Hounsfield unit</td>
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<tr>
<td>IDM</td>
<td>inverse difference moment</td>
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<tr>
<td>ITM</td>
<td>interterritorial matrix</td>
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<td>OA</td>
<td>osteoarthritis</td>
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<td>PG</td>
<td>proteoglycan</td>
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<td>µCT</td>
<td>micro-computed tomography</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>PMA</td>
<td>phosphomolybdic acid</td>
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<tr>
<td>PTA</td>
<td>phosphotungstic acid</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SRCT</td>
<td>synchrotron-based micro-computed tomography</td>
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<td>Tb.N.</td>
<td>trabecular number</td>
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<td>Tb.Th.</td>
<td>trabecular thickness</td>
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<td>TKA</td>
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<tr>
<td>VIF</td>
<td>variance inflation factor</td>
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<td>VOI</td>
<td>volume of interest</td>
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List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:


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1 Introduction

Osteoarthritis (OA), is a group of degenerative joint disorders with heterogenic origin but similar biologic, morphologic and clinical outcomes, i.e., defective articular cartilage integrity and related changes in subchondral bone and joint margins (Cooper, Javaid, & Arden, 2015). OA commonly occurs in hand, knee, spine and hip joints, although it can also be present in any synovial joint (Buckwalter & Martin, 2006). Clinical symptoms of OA are characterised by synovial joint pain and dysfunction, and the diagnosis is often supported by radiographical, arthroscopic and/or magnetic resonance imaging (MRI) findings of the altered joint or tissue structure (Bijlsma, Berenbaum, & Lafeber, 2011; Glyn-Jones et al., 2015; Madry et al., 2016). Currently, the treatments for OA are limited to pain alleviation and ensuring the mobility of the joint by medication (e.g., nonsteroidal anti-inflammatory drugs) and therapeutic exercise. In late-stage OA, the only effective treatment option is joint arthroplasty (Working group appointed by the Finnish Medical Society Duodecim and the Finnish Orthopaedic Association, 2014). As OA is ranked among the diseases with the highest years lived with disability (Vos et al., 2012), it also has high socioeconomic impact. The amount of direct and indirect costs of OA to society vary between demographics (Chen, Gupte, Akhtar, Smith, & Cobb, 2012). Nevertheless, the costs are not only substantial to societies, but also increasing, as the OA risk factors, mainly age and obesity, in populations are increasing (Bitton, 2009; Chen et al., 2012).

The OA risk factors can be divided into person-level risk factors (e.g. age, obesity, and genetics) and joint-level factors (e.g. injury, malignment and abnormal loading) (Johnson & Hunter, 2014). However, whether these factors can be associated with specific OA phenotypes needs to be elucidated. It has been suggested that not only may the progress of OA differ with different sub-groups or phenotypes of OA (Deveza et al., 2017), but there can also be interrelated associations between the different phenotypes (Bierma-Zeinstra & van Middelkoop, 2017). As OA's heterogeneous nature generates difficulties in developing prevention and treatment techniques, further research on the progression of OA is required.

Structural alterations are strongly associated with symptomatic OA (Hunter, Guermazi, Roemer, Zhang, & Neogi, 2013; Karsdal et al., 2015). OA affects multiple tissues in the joint, and degeneration of the articular cartilage and subchondral bone alterations are the primary structural alterations with OA progression (Buckwalter & Martin, 2006). Improvement of clinical and research
imaging modalities would help to understand the aetiologies and phenotypes of OA and allow identification of early OA biomarkers. This would enable more effective measurement of OA progression and severity, which is urgently needed for monitoring in the development of prevention techniques and treatments for OA.

Conventional radiography, MRI, ultrasound and computed tomography (CT) are often-used modalities in OA research (Hayashi, Roemer, & Guermazi, 2016). Of these imaging modalities, only MRI and CT are applicable for imaging OA features in both bone and cartilage in 3D. MRI has been called the “key imaging tool” for OA research; however it is lacking in imaging bone architecture and resolution when compared to CT. For OA research in clinical setup, native CT is excellent for imaging cortical bone and calcifications of soft tissues in vivo, and with the use of contrast agents, CT is the most accurate method for evaluating superficial and focal cartilage damage with high-contrast and spatial resolution (Hayashi et al., 2016).

In vitro and in vivo micro-computed tomography (µCT) imaging of osteochondral tissues has been widely used in pre-clinical and basic OA research (Kuyinu, Narayanan, Nair, & Laurencin, 2016). The recent development of contrast agents has introduced non-destructive 3D imaging of soft tissues with µCT at histological resolution (Metscher, 2009a; Metscher, 2009b). The OA research with µCT imaging has been focused mainly on animal models, but the developmental of µCT imaging methods has potential to provide 3D histopathological tools for OA research.

The need to evaluate OA severity in basic OA research has resulted in the development of several histopathological grading systems (Mainil-Varlet et al., 2010; Mankin, Dorfman, Lippiello, & Zarins, 1971; Pritzker et al., 2006). These grading systems are based on visual evaluation of the degeneration of the articular cartilage and subchondral bone from stained histological sections imaged using light microscopy. The histological sectioning of the tissue limits the evaluation of the 3D structure of the tissue, as the sectioning can produce sample preparation artefacts and the 2D sections are not representative of the 3D structure. Micro-scale 3D imaging of the intact osteochondral tissues would ease these limitations. Furthermore, the advantage of digitised volumes would help in developing automated evaluation systems which are more prone to human error.

In addition to µCT, other potential 3D imaging modalities for osteochondral tissues include optical coherence tomography (O’Malley & Chu, 2011), micro-MRI (Xia, 2013), confocal microscopy (Youn, Choi, Cao, Setton, & Guilak, 2006), photoacoustic tomography (Yao, Sobel, & Huabei, 2011), and scanning electron
microscopy (SEM) (Hughes, Archer, & ap Gwynn, 2005). While these methods try to provide 3D structures and composition of osteochondral tissues with minimal destructiveness, they have other limitations. Some of these methods are either only topographical (SEM) or are limited in tissue depth (confocal microscopy). Furthermore, of these methods, µCT is the only 3D-imaging modality which can reliably visualise micro-structures at a 1-10 µm resolution, from relatively larger sample volumes (a few mm²).

This thesis focuses on introducing potential methods for quantifying OA-related structural changes in articular cartilage and subchondral bone with in vitro µCT imaging.
2 Articular cartilage and subchondral bone

Articular cartilage is aneural, avascular and alymphatic connective tissue in the ends of diarthrodial joints (Buckwalter & Mankin, 1998b; Huber, Trattnig, & Lintner, 2000; Mow, Ratcliffe, & Poole, 1992). The main function of articular cartilage is to provide a lubricated, low-frictional surface for articulation and load transmission of the joint (Sophia Fox, Bedi, & Rodeo, 2009). In the knee joint, articular cartilage covering patella and condyles of tibia and femur is typically 1-4 millimetres thick (Shepherd & Seedhom, 1999). Articular cartilage has highly depth-dependent anisotropic structure and distribution of tissue constituents (see Fig.1).

![Fig. 1. A: Schematic presentation of the knee joint anatomy in sagittal view. B: Schematic presentation of the depth-wise distribution of constituents of the extracellular matrix (ECM), i.e. collagen, proteoglycan and water, and chondrocyte density (cells/mm³) in uncalcified cartilage (from cartilage surface to the interface)](image-url)
between calcified tissue and uncalcified cartilage, called the tidemark). The colour bar at the bottom indicates the relative contents. C: Schematic presentation of the structure of osteochondral tissue. Both the collagen network orientation (blue lines) and the chondrocyte (white ellipsoids) shape, orientation and volume have depth-wise variation in articular cartilage. Between the subchondral bone and uncalcified cartilage lies the calcified cartilage layer.

Underneath the articular cartilage lies bone tissue. The main functions of bone are to support and protect soft tissues, enable locomotion, act as mineral reservoir, and accommodate bone marrow. Bone is a highly mineralised and adaptive tissue: its adult bone cells sense mechanical load and respectively control bone mass and microstructure based on the mechanical use. (Buckwalter, Glimcher, Cooper, & Recker, 1996a; Buckwalter, Glimcher, Cooper, & Recker, 1996b)

2.1 Composition and structure of articular cartilage

Articular cartilage consists of the extracellular matrix (ECM) and sparsely distributed specialised cartilage cells, i.e. chondrocytes. The ECM consists of collagen, proteoglycans (PGs), water and lesser amounts of other glycoproteins, lipids and non-collagenous proteins (Buckwalter & Mankin, 1998b). Collagens in articular cartilage are mostly type II, although minor amounts of other types of collagens (e.g. VI, IX, and XI) are also present in the tissue (Buckwalter & Mankin, 1998b). Collagen fibrils form a network providing tensile stiffness and strength to the articular cartilage by constraining the swelling pressure of PGs (Han, Grodzinsky, & Ortiz, 2011; A. I. Maroudas, 1976). In addition, PGs contribute to resistance to shear (Jin & Grodzinsky, 2001) and compressive (Williamson, Chen, & Sah, 2001) deformations. PGs are formed by core protein and highly anionic glycosaminoglycan (GAG) chains (Knudson & Knudson, 2001). The high negative charge of the GAG chains, due to the high amounts of negatively charged sulfate and carboxyl groups, attract counterions (e.g. Na⁺) and give rise to Donnan osmotic pressure favouring tissue hydration (Lesperance, Gray, & Burstein, 1992; A. Maroudas, 1968).

Chondrocytes maintain and produce the constituents of ECM. Chondrocyte shape, density, volume fraction, surface area and volume vary along with cartilage depth (Hunziker, Quinn, & Häuselmann, 2002). The surrounding ECM can be divided into the pericellular, territorial and interterritorial matrices based on the circumferential variation of the matrix components around the chondrocytes. In the close vicinity of the chondrocytes is an approximately 2 µm thick pericellular
matrix (Poole, Flint, & Beaumont, 1987). This is made up of a high amount of collagen type VI compared to the rest of the cartilage tissue, and is enriched with hyaluronan, sulfated proteoglycans, biglycan and a range of matrix glycoproteins (Poole, 1997). The pericellular matrix and chondrocyte form a structural and functional unit: a chondron. The territorial matrix surrounding chondrons contain higher amounts of chondroitin sulfate-rich proteoglycans compared to the interterritorial matrix, which, in turn, contains higher amounts of keratin sulfate compared to the territorial matrix (Poole, 1997). The collagen content also varies as the distance to the chondrons increases, e.g. the highest amounts of collagen VI (Poole, Gilbert, Herbage, & Hartmann, 1997; Wotton, Jeacocke, Maciewicz, Wardale, & Duance, 1991) and IX (Poole, Ayad, & Gilbert, 1992) are at the pericellular matrix, with small quantities in the territorial matrix. The interterritorial matrix consists mostly of the thickest collagen II bundles with minor or non-existent amounts of collagen VI and XI (Poole, 1997).

The structure of healthy hyaline articular cartilage is inhomogeneous, and the distribution of the ECM constituents, collagen fibril orientation, and chondrocyte orientation, density, and morphology vary with the depth of the tissue (see Fig. 1). The hyaline cartilage can be structurally divided depth-wise into the surface, middle, and deep zones. In the surface zone (or tangential zone) the fine collagen fibres are densely aligned parallel to the surface of the tissue. The chondrocytes are more elongated and oriented parallel to the articular cartilage surface. The surface zone has the highest water and collagen contents and lowest PG content when compared to the other zones. The middle zone (or transitional zone) has more rounded chondrocytes, and the collagen fibrils are randomly oriented and thicker compared to the surface zone. The water content decreases and PG content increases gradually with the depth of the tissue. Thus, the deep zone (radial zone) has the lowest water content and highest PG density. Collagen fibrils form thicker bundles and are organised radially. The deep zone has the highest cell density and the chondrocytes form columns of multi-chondrocyte chondrons, which are parallel to the collagen fibril bundles. These bundles are anchored to the calcified cartilage through an irregular calcification interface called the tidemark. (Buckwalter & Mankin, 1998b; Sophia Fox et al., 2009)

Calcified cartilage transmits forces and acts as a solute transport barrier between the uncalcified hyaline cartilage and subchondral bone (Oegema, Carpenter, Hofmeister, & Thompson, 1997). Compared to uncalcified cartilage, calcified cartilage contains higher amounts of type X collagen and has vascular
channels originating from the cortical bone (Madry, van Dijk, & Mueller-Gerbl, 2010).

2.2 Composition and structure of subchondral bone

Bone tissue consists of an organic and inorganic matrix. Organic matrix makes up 20-30% of the wet weight of the bone. It consists of primarily type I collagen along with small amounts of collagen types V and XII, and non-collagenous glycoproteins and bone-specific PGs. The organic component in bone gives it its compressive strength. The inorganic matrix acts as an ion reservoir and provides bone with its strength and stiffness. The inorganic matrix, or mineral component, makes up 55% of the bone tissue and consists mainly of hydroxyapatite crystals and amorphous calcium phosphate. (Buckwalter et al., 1996a; B. Li & Aspden, 1997)

In mature bone tissue, 90% of the bone cells are osteocytes, which are mechanosensory cells entrapped inside the bony matrix in a space called the lacuna (Buckwalter et al., 1996a). Osteocyte cytoplasmic processes are connected to other cells by channels called canaliculi. Osteocytes maintain the bone matrix, but also take part in remodelling and bone turnover in the role of modulator of the activity (Bonewald, 2010; Stains & Civitelli, 2005). Bone cells which are more directly responsible for the remodelling and bone turnover are called osteoblasts, mononucleate cells forming new bone, and osteoclasts, multinucleate cells resorbing bone matrix. Mature bone also contains bone-lining cells, which attract osteoclasts, and undifferentiated mesenchymal cells. These mesenchymal cells are activated to differentiate into osteoblasts e.g. in the case of bone fracture (Buckwalter et al., 1996a; Raggatt & Partridge, 2010).

Bone can be morphologically separated into the stiffer and more compact cortical bone covering the outer layers and the inner more porous trabecular bone filled with bone marrow (Buckwalter et al., 1996a). Subchondral bone, separated from calcified cartilage by the cement line, entails the cortical endplate, also known as the subchondral bone plate, and the supporting trabecular bone underneath the plate. Subchondral bone provides a supporting structure for the cartilage and acts as the joint’s shock absorber during mechanical loading (Madry et al., 2010). Furthermore, the subchondral bone plate provides a route for nutrients to reach the cartilage from the bone marrow. The plate has channels infiltrating from the trabecular bone to the calcified cartilage, providing a route to the vessels and nerves (Clark, 1990; Madry et al., 2010).
2.3 Osteoarthritis

Osteoarthritis (OA) causes joint degeneration, including progressive loss and changes to the composition of articular cartilage and calcified cartilage, remodelling and sclerosis of subchondral bone, osteophyte formation, muscle weakness, and inflammation of the synovium tissue and tendon (Buckwalter & Mankin, 1998a; Buckwalter & Martin, 2006; G. Li et al., 2013; Madry et al., 2016; Pritzker, 2003).

The heterogenic origin of the structural tissue alterations of OA has led to identification of structural phenotypes, *inter alia* cartilage-driven (Sandell & Aigner, 2000) and bone-driven (Karsdal et al., 2015) OA progression. These theories are intuitive, as the primary structural changes with the OA progression are degeneration of articular cartilage and subchondral bone remodelling (Buckwalter & Martin, 2006).

In articular cartilage, the first structural changes caused by OA include an increase of water content, decrease of proteoglycan aggregation, and depletion of proteoglycan content. Disruptions in the ECM can be present in the form of fibrillation of the superficial matrix, even though collagen content in the tissue has not yet decreased. Swelling of the cartilage can be present due to the alterations in collagen fibril integrity. Since these structural changes affect the stiffness of the tissue, they may increase the vulnerability of the tissue to further mechanical stresses. The chondrocytes respond to the structural and compositional changes of the articular cartilage. The chondrocyte activity, synthesis of ECM macromolecules and proliferation increases with tissue degradation. This leads to chondrocyte clustering and hypertrophy, which are often the early OA features in histopathological OA grading with cartilage surface irregularities and PG depletion. Failure to stabilise and repair the tissue leads to progressive loss of the articular cartilage matrix. The fibrillation of the surface progresses to clefts reaching through the surface zone, eventually leading to further matrix loss, increase in chondrocyte clustering, and PG depletion. As the hyaline cartilage is completely eroded, subchondral bone and calcified cartilage are vulnerable to mechanical stresses while increasing subchondral bone remodelling and cyst formation. (Buckwalter & Martin, 2006; Mankin et al., 1971; Pritzker et al., 2006)

Traditionally, progressive articular cartilage degeneration with subchondral bone sclerosis is considered a hallmark of OA (Burr & Gallant, 2012; Henrotin, Pesesse, & Sanchez, 2012; G. Li et al., 2013). However, a range of studies suggest that subchondral bone turnover has a role in cartilage degeneration (Bellido et al.,
Furthermore, this is supported by evidence on the association between cartilage degeneration and the subchondral bone structure (Chou et al., 2013; Finnilä et al., 2017; Yan et al., 2018). Structural alterations in the subchondral bone during the progression of OA include an increase in the bone volume and trabecular number, thickening of the subchondral bone plate, changes in the shape and orientation of the trabeculae, decrease in bone marrow spacing and trabecular separation, cyst formation and bone marrow oedema-like lesions in the subchondral bone, and hypomineralisation of the subchondral bone (Chou et al., 2013; Finnilä et al., 2017; G. Li et al., 2013; Yan et al., 2018). Furthermore, osteocyte morphology has been shown to alter in OA, and this is considered to be related to subchondral remodelling (Jaiprakash et al., 2012).
3 Pre-clinical microscopy of osteochondral tissues

As described earlier, the main function of the osteochondral tissues is to maintain and provide functionality of the joints, and disruption of functionality (e.g. due to osteoarthritis) means that alterations in composition and structure are present. Thus, structural and compositional parameters are obvious measures for the “healthiness” of these osteochondral tissues, i.e. articular cartilage and subchondral bone. Due to the anisotropic nature and microscopic size of the smallest key features (e.g. cartilage zonal anisotropy see section 2.1), the pre-clinical microscopic imaging of osteochondral tissues is a crucial element in OA research. Various imaging modalities can provide qualitative and quantitative information on these tissues, but perhaps the “gold standard” is the histopathological evaluation that is often used as a reference when developing new micro-scale imaging modalities. Here we focus mainly on the reference methods used in this thesis, by providing a short description of these modalities before the introduction of computed tomography (see section 4).

Histological staining combined with light microscopy is the “gold standard” in imaging of the composition and morphology of articular cartilage and subchondral bone. The conventional histology requires a decalcification process combined with fixation of the tissue (for preservation) prior to sectioning a few micro-meter thick sections (Luna, 1968; Schmitz, Laverty, Kraus, & Aigner, 2010). The histological slices can be stained depending on the compositional or structural feature of interest. One popular example is Safranin O-stain of articular cartilage, which by its cationic component binds to the anionic molecular chains of proteoglycans (Luna, 1968; Schmitz et al., 2010). After the staining, light microscopy can be used for qualitative assessment, e.g. grading the severity of OA (Pritzker et al., 2006).

Polarised light microscopy (PLM) can be used to determine the collagen fibril orientation and the retardance of osteochondral tissues (Mittelstaedt, Xia, Shmelyov, Casciani, & Bidhanapally, 2011; Spiesz, Kaminsky, & Zysset, 2011; Xia, 2008), the latter parameter of which also depends, in addition to orientation, the collagen fibril thickness and packing density of the sample. PLM is conducted on histological sections, often without the staining procedure, but some stains (e.g. Picrosirius Red) can enhance the birefringence of the collagen fibrils (Junqueira, Bignolas, & Brentani, 1979). Basically, the PLM setup consists of a light microscopy system with two polarising filters at a 90-degree angle to each other. When a sample with birefringent properties (e.g. collagen) is positioned between...
the polarisers it will split the light into two polarisation components with different velocities. Measurement of the phase difference (or delay) between the two polarising components contains birefringent information for the sample, which can be determined from the retardance (Xia, 2008). Nowadays in practice, more automated processes have been adopted in order to provide simultaneous gathering of birefringence from multiple quadrants (Kaminsky, Gunn, Sours, & Kahr, 2007).

Fourier Transform Infrared Imaging (FTIRI) provides compositional information on the articular cartilage and subchondral bone. FTIRI is based on measuring the infrared (IR) absorption in the sample at specific wavelengths. In general, different molecular compositions (or specific chemical bonds) absorb IR at different wavelengths; thus, the composition of the object can be determined when the measurement is conducted at multiple wavelengths. FTIRI can gather multiple wavelengths simultaneously, as the broadband light source combined with an adjustable interferometer enables different wavelengths modulated at different rates to be gathered. The resulting raw data is called an interferogram, which contains information on the light absorption at the specific mirror position. Applying Fourier Transform to the interferogram results in a spectrum of different wavelengths containing compositional information for the measured sample location. FTIRI is often conducted on histological sections from multiple locations to obtain a spectral “map”. In this kind of spectral image, each pixel contains its own absorption spectrum. In post-processing the spectrum, e.g. by calculating the areas of specific wavelength bands of the spectrum which correspond to the specific molecular composition, distribution maps and the amount of specific molecular bonds can be quantitated from the samples. (Boskey & Pleshko Camacho, 2007; Griffiths, De Haseth, & Winefordner, 2007; Xia, 2008)

In addition to these imaging modalities, there are multiple other modalities capable of producing the microscopic composition and/or structure of the osteochondral tissues. For example, micro-MRI can provide compositional and structural information on articular cartilage (Xia, 2013; Xia, 2008) and structural information for bone (Hipp, Jansujwicz, Simmons, & Snyder, 1996; Wehrli, 2007). Some imaging modalities are more specific to a certain structure, such as topography of the articular cartilage surface (SEM and transmission electron microscopy (TEM)) (Hughes et al., 2005; Xia, 2008) and subchondral bone (optical coherence tomography) (Bykov et al., 2016), or chondrocyte morphology (confocal microscopy) (Youn et al., 2006).
4 Computed tomography

4.1 Basic principles of computed tomography

In computed tomography (CT) imaging, X-ray projection images are acquired in several angular positions around the object subjected to the imaging. The change in the X-ray intensity as it travels through the imaging object is recorded on a projection image. Acquiring projection images from several angles provides attenuation profiles, which have to be subjected to inverse transformation to determine the spatial distribution of attenuation coefficients within the object. This inverse transformation, or more commonly image reconstruction, utilises inter alia Fourier-domain, back-projection, and iterative algorithms. The image reconstruction results in 3D volumes or 2D cross-sectional images where each voxel/pixel value describes an X-ray attenuation value. (Dowsett, Kenny, & Johnston, 2006; Kalender, 2011)

The interactions between X-rays and matter always leads to attenuation of the X-ray beam because the interaction processes include absorption and/or scattering of the X-ray photons (Dowsett et al., 2006). Furthermore, with poly-energetic X-rays (in practice with all X-ray tube sources) the lower-energy photons scatter and absorb “faster” than higher-energy photons when travelling through the objects, creating a phenomenon called beam-hardening (Dowsett et al., 2006). These interactions are dependent on the object’s properties (atomic number, density and thickness) and the X-ray beam quality (filtration and effective beam energy). The linear attenuation coefficient describes the overall attenuation of the X-rays and, thus, is related to all the X-ray interaction processes and their dependencies. (Dowsett et al., 2006) In addition to the linear attenuation coefficient, other CT values have been used to disregard some of these dependencies, for instance, the mass-attenuation coefficient which is invariant to the object density (Dowsett et al., 2006). The most commonly used presentation of the tissue linear attenuation coefficient in biomedicine and clinical radiology is the Hounsfield unit (HU), which provides a quantitative and universally available scale for CT values (Hounsfield, 1973). HU values are defined by the linear attenuation coefficient of water with equation 1:

\[ HU = 1000 \times \frac{\mu - \mu_w}{\mu_w}, \]  

(1)
where $\mu_o$ is the linear attenuation coefficient of X-ray in the material and $\mu_w$ is the attenuation coefficient of water. Air corresponds to a HU value of -1000 as the general approximation for the attenuation coefficient of air is 0 (Kalender, 2011). In addition to the fixed HU value of water (HU: 0), HU value ranges for common tissue types (e.g. soft tissues +100 to +300, fat -120 to -85, bone +300 to +3000) are often presented in the literature (Dowsett et al., 2006; Kalender, 2011). To maintain focus on the topic of this thesis, further information on the basics of X-ray physics and CT imaging can be found in the literature (Dowsett et al., 2006; Kalender, 2011).

### 4.2 Micro-computed tomography in cone-beam geometry

Cone-beam geometry is one of the measurement and reconstruction geometries in CT imaging, in which the X-rays spread out from the focal spot of their source to the detector in the shape of cone. All CT applications which use cone-beam reconstruction can be defined as cone-beam computed tomography (CBCT); thus CBCT is not limited to one detector or source properties. (Kalender, 2011)

One application of CT using cone-beam geometry is micro-computed tomography ($\mu$CT). In general, $\mu$CT is a CT application which reaches micrometre-scale spatial resolution. To reach high spatial resolution, $\mu$CT systems utilise a micro-focus X-ray tube and provide a focal spot diameter of a few $\mu$m, and geometrical and/or objective (lens) magnification. (Gregor et al., 2012; Kalender, 2011)

Synchrotron-based micro-computed tomography (SRCT) utilises synchrotron-generated X-ray beams as a source. The main advantages of SRCT, compared to conventional $\mu$CT imaging, are the high signal-to-noise ratio (SNR) with low scan times in high resolution due to the high photon flux and the utilisation of phase contrast (Ma et al., 2016; Peyrin, Dong, Pacureanu, & Langer, 2014). Furthermore, the lack of beam-hardening artefacts due to the monochromatic X-ray beam (Peyrin et al., 2014) allows more accurate mineral density determination of bone with SRCT compared to the conventional $\mu$CT (Kazakia, Burghardt, Cheung, & Majumdar, 2008).

$\mu$CT has become a gold standard for high-resolution bone imaging for in vitro and in vivo studies (Müller, 2009). Desktop $\mu$CT applications provide quantification of trabecular bone microstructure (Bouxsein et al., 2010; Odgaard, 1997) and cortical bone microstructure (Bouxsein et al., 2010), and good approximation for tissue mineral density (Kazakia et al., 2008; Postnov, 2009).
Vinogradov, Van Dyck, Saveliev, & De Clerck, 2003). Furthermore, with SRCT the sub-micro-structures of bone, e.g. osteocyte lacunae morphology, can be easily/reliably quantified (Peyrin et al., 2014).

4.3 Contrast-enhanced micro-computed tomography

X-ray contrast agents are utilised in CT to provide contrast between the soft tissues by increasing the X-ray attenuation of the tissue or structure of interest. Thus, it is recommendable that the contrast agents contain chemical elements with a high atomic number, e.g. iodine, gadolinium, or tungsten, and a molecular body with an affinity to a tissue or cell type. (Lusic & Grinstaff, 2013; Yu & Watson, 1999) The introduction of contrast agents to μCT imaging has enabled quantitative pre-clinical in vivo and ex vivo contrast-enhanced μCT (CEμCT) imaging of soft tissue structures at a resolution equivalent to histology (Metscher, 2009a; Metscher, 2009b; Schambach, Bag, Schilling, Groden, & Brockmann, 2010).

CEμCT of articular cartilage can be used to quantify the GAG content of the articular cartilage (Bansal et al., 2011; Cockman et al., 2006; Joshi, Bansal, Stewart, Snyder, & Grinstaff, 2009; Mittelstaedt & Xia, 2015; Palmer, Guldberg, & Levenston, 2006; Silvast, Jurvelin, Aula, Lammi, & Töyräs, 2009; Xie, Lin, Levenston, & Guldberg, 2009; Yoo et al., 2011). The highly negative GAG content generates a fixed charge density (FCD) in the articular cartilage, which repulses anionic molecules and attracts cationic molecules based on Donnan’s equilibrium. Consequently, contrast agents designed for the evaluation of GAG distribution of articular cartilage distribute either inversely with anionic contrast agents (Mittelstaedt & Xia, 2015; Palmer et al., 2006; Silvast et al., 2009; Xie et al., 2009; Yoo et al., 2011), or proportionally with cationic contrast agents (Bansal et al., 2011; Cockman et al., 2006; Stewart et al., 2013), to the GAG content based on the charge of the contrast agent molecule. Cationic contrast agents have been shown to have higher association with the GAG content compared to anionic contrast agents (Bansal et al., 2011; Cockman et al., 2006).

The FCD of the cartilage tissue is altered by the pH of the environment (Lesperance et al., 1992; Loret & Simões, 2010). The collagen molecule has an amino group \( \text{NH}_3^+ \), called the N-terminus, and a carboxyl group \( \text{COO}^- \), called the C-terminus. At neutral pH, the charges of the collagen are in balance; however, due to these amino- and carboxyl groups, the collagen molecule becomes positively charged at low pH and negatively charged at high pH (Loret & Simões, 2010). Unlike collagen, the net charge of PGs stays negative regardless of the
environmental pH (Loret & Simões, 2010). Collagen-specific histological stains, e.g. Masson’s trichrome stain, utilise pH manipulation of the articular cartilage to lower the pH, thus making the net charge of collagen positive, enabling columnic binding of anionic “bridge” molecules, e.g. phosphotungstic acid (PTA) or phosphomolybdic acid (PMA) to the collagen network (Constantine & Mowry, 1968; Puchtler & Isler, 1958; Quintarelli, Zito, & Cifonelli, 1971). As PTA and PMA contain molecules with high atomic numbers, they would attenuate X-rays and thus are suitable as contrast agents in CEµCT studies. In fact, the use of PTA and PMA as contrast agents in in vitro CEµCT protocols for the field of developmental biology has been suggested (Metscher, 2009a; Metscher, 2009b).

4.4 Textural analysis and computed tomography

Currently, spatial resolution of clinical CT is not at a level where subchondral bone architecture is quantifiable: trabecular structure has been reported to be quantifiable up to 100 µm voxel side length (Isaksson et al., 2011). The insufficient resolution leads to a partial volume effect where objects (e.g. trabeculae) smaller than the voxel size result in partial signal intensity. As this limits the direct quantification of these “sub-resolution” structures, voxel intensities from a CT volume still have an association to some structural parameters. For example, the averaged grey-level values of the clinical resolution CT and microstructural trabecular bone morphometrics have been reported to have a significant correlation (Hsu et al., 2014).

As the microstructural elements affect the voxel intensities and their spatial arrangement, i.e. image texture, advanced computer vision methods can be applied to quantify variations in the voxel intensities and the spatial arrangement. This quantification process, or texture analysis, can be conducted using several types of methods (i.e. structural, model-based, transform-based and statistical) (Bharati, Liu, & MacGregor, 2004). These methods have been extensively applied in the analysis of bone morphometrics from 2D radiographs (Chappard et al., 2001; Hirvasniemi et al., 2015; Hirvasniemi et al., 2017; Messent, Buckland-Wright, & Blake, 2005; Pothuaud, Carceller, & Hans, 2008). Application of these methods for volumetric CT data sets some limitations, but e.g. grey-level co-occurrence matrix (GLCM) based texture analysis (Haralick, Shanmugam, & Dinstein, 1973; Haralick, 1979) methods have shown promising results in the quantification of bone structure from CT data (Diederichs et al., 2009; Mookiah et al., 2018; Nagarajan et al., 2015; Shirvaikar, Huang, & Dong, 2016). One aim of this thesis is to apply the GLCM...
method to cone-beam CT and μCT. A more detailed description of the method can be found in section 6.5.4.
5 Aims of the Thesis

The aim of this thesis is to create and validate novel μCT methods which can be used to quantify osteochondral micro-structures related to osteoarthritic changes within articular cartilage and subchondral bone. More specifically, the aims for the individual sub-studies were:

1. Introduce and test two potential contrast agents, PTA and PMA, for the quantification of collagen content distribution in articular cartilage with CEμCT.
2. Test the specificity of PTA to articular cartilage constituents in the CEμCT setup.
3. Investigate the micro-scale distribution of cationic contrast agent CA4+ in articular cartilage for the GAG-specific CEμCT method.
4. Investigate the feasibility of histogram and texture-based analysis to quantify sub-resolution bone morphometrics related to OA from μCT and clinical resolution CBCT.
6 Materials and Methods

The materials and methods used in this thesis are described below. The original publications for which the materials and methods have been used are indicated with Roman numerals corresponding to the list of original publications.

6.1 Samples

6.1.1 Equine samples (I)

Equine samples were used in the first sub-study (I). Three equine joints were acquired from the local abattoir (Veljekset Rönkä Oy, Kemi, Finland). Twelve osteochondral cylinders, with core diameter of 6 mm, were extracted from the anterior medial ($n=3$), posterior medial ($n=3$), anterior lateral ($n=3$) and posterior lateral ($n=3$) of proximal phalanxes. Each core was sectioned into three pieces. Two of the three pieces were subjected to CEµCT, one with PTA and one with PMA. The last piece of the three was reserved for histological slicing and Fourier infrared imaging (FTIRI) analyses as a reference for the cartilage composition.

6.1.2 Bovine samples (II)

Bovine samples were used in the second sub-study (II). Nine right hind limbs of bovines were acquired from the local abattoir (Veljekset Rönkä Oy, Kemi, Finland) and stored in a freezer at -20 °C prior to the experiments. After thawing, three adjacent osteochondral cores, with core diameter of 4.8 mm, were extracted from the upper quadrant of each patella. From each patella, the three adjacent cores were divided into a collagenase treatment, chondroitinase ABC treatment, and untreated (control) group. Prior to the enzymatic treatments, the cores were cut into four pieces.

To study the effect of articular cartilage constituents, enzymatic degradation of collagen and proteoglycan content were conducted on two adjacent cores, already sectioned to four pieces, while the third adjacent core was left untreated. The control samples were subjected to CEµCT and reference analyses. For the PG degradation, the pieces were immersed in 0.1 U/ml chondroitinase ABC solution for 44 hours at 37 °C, 5% CO₂, which degraded the chondroitin/dermatan sulfate and hyaluronan glycosaminoglycans (GAGs). The pieces subjected to collagen
degradation were immersed in 30U/ml collagenase solution for 50 hours at 37 °C, 5% CO₂.

After the enzymatic treatments, the first and second pieces were subjected to CEμCT with PTA and Hexabrix®, respectively. The third and fourth pieces were subjected to the reference methods: FTIRI and biochemical analysis, respectively.

6.1.3 Human Samples (I, III, & IV)

The first, third and fourth sub-study utilised human samples harvested from total knee replacement patients (TKR). TKR samples were collected under approval of the ethics committee of Northern Ostrobothnia Hospital District (permit no: 78/2013) with the written consents of the patients. In addition to samples gathered from TKR patients, samples from human cadavers were utilised to produce the data for the fourth sub-study. Samples from cadaveric patients were harvested under the approval of the research ethics committee of the Northern Savo Hospital District (permit nos: 58/2013 and 134/2015).

For the first sub-study, samples from two TKR patients were used (2 males, aged 58 and 77 years). From each patient one osteochondral core, with diameter 4.8 mm, was drilled from the femoral and one from the tibial joint surface of the knee joint. The cores were cut into two quarters and one half. The halves were subjected to conventional histology with Masson’s trichrome stain and FTIRI analysis. One quarter was subjected to CEμCT with PTA.

For the third sub-study, two sample sets were selected. The first sample set for analysing the diffusion of CA4+ in articular cartilage consisted of samples harvested from three TKR patients (one female, two males, age range 56-77 years). From each patient four adjacent cores, with diameter 2 mm, were drilled and three of the four adjacent cores were exposed to different concentrations (3, 6, and 24 mgI/ml) of CA4+ prior to CEμCT. The fourth adjacent core was subjected to histology with Safranin O and Toluidine blue staining. The second sample set consisted of 14 TKR patients (10 females, four males, age range 51-86 years). From each patient, two adjacent osteochondral cores, with diameters 2 mm and 4mm, were extracted from the tibial surface of the knee joint. The 2mm core was subjected to CEμCT with CA4+ and the 4mm core was subjected to histological evaluation with Safranin O-staining.

For the fourth sub-study, a total of 56 cores were extracted from the tibial plateaus and femoral condyles of TKR patients and cadavers. Forty-nine of these cores were extracted from the tibial plateaus of two TKA patients (one tibial plateau
per patient) and two cadavers (both tibial plateaus). The cores were extracted from eight areas of the tibial plateau (see Figure 2). To ensure high variability in OA severity, an additional seven cores were extracted from TKA patients (N=7). Sample exclusions and sub-grouping based on the core location is shown in Figure 2. Prior to imaging, the samples were immersed in 4% saline buffered formaldehyde. The samples were imaged in a container filled with fixation media and cellulose to prevent the sample moving inside the container during imaging.

**6.2 Contrast-enhanced micro-computed tomography (CEµCT) of articular cartilage (I, II, III)**

**6.2.1 CEµCT with phosphotungstic acid and phosphomolybdic acid (I, II)**

Prior to the staining of the osteochondral samples with PTA or PMA, the samples were fixed in 10% neutral-buffered formalin (4% formaldehyde) for 5 days. The PTA and PMA solutions were created by mixing 1% w/v of the PTA or PMA hydrate in 70% ethanol.

For the first sub-study, adjacent quarters from equine osteochondral cores were imaged with µCT at multiple time points during PTA and PMA immersions. The samples were scanned with a Skyscan 1176 (Bruker microCT, Kontich, Belgium).
µCT at predefined time points of 0, 18, 36, 54, 72, 90, 180 and 270 hours of immersion in the contrast agent. Prior to the µCT scan, the sample was removed from the contrast agent solution, rinsed in 70% ethanol and sealed in a container with cotton balls moistened with 70% ethanol to prevent drying during the scan. The µCT scan was conducted with 80 kV, 300 mA, 658 projections, exposure 1050 ms/frame, average of two frames per projection, isotropic voxel side length of 8.7 µm, and an additional bilayer filter consisting of 0.5 mm aluminium and 0.038 mm copper. The projections were reconstructed with Nrecon software (v. 1.6.9, Bruker microCT). Based on the equine µCT scans, the CEµCT protocol was selected for human samples. The human samples were immersed in PTA for 36h, removed from the solution and prepared for the µCT scan as with the equine samples. The µCT scan of human samples was performed using a Nanotom 180NF (Phoenix X-ray Systems/GE,) with 80kV, 100µA, 1200 projections, exposure 750 ms/frame, average of seven frames per projection, 3.2 µm voxel side length and without additional X-ray filtration. The projection data was reconstructed using datos|x software (v. 1.3.2.11, GE Measurement & Control Solutions/Phoenix X-ray, Fairfield, CT, USA).

The reconstructed 3D datasets of equine and human samples were co-registered (with 6 degrees of freedom) and resliced to correspond to the histological slicing direction using Automated Image Registration software (v. 5.3.0 University of California, Berkeley, CA, USA). One image slice with a thickness of 8.7 µm per sample was selected close to the location where the histological slices were sectioned and depth-wise profiles of the image intensities were analysed.

For the second sub-study, quarters of bovine osteochondral core were subjected to CEµCT with PTA. The enzymatically treated, and control group samples were fixed for 5 days and stained for 36 h in 1% w/v PTA solution as described above. Similarly as in the first sub-study (I), samples were prepared and sealed in a container with cellulose and 70% ethanol for the µCT scan. The CEµCT scan was conducted with a Skyscan 1176 (Bruker microCT) with 40kV, 600µA, 1200 projections, exposure 200 ms/frame, average of two frames per projection, isotropic voxel side length 17.4 µm, and without additional filtration. The projection data was reconstructed using Nrecon software (v. 1.6.9, Bruker microCT) as in the first sub-study. The reconstructed 3D datasets were similarly co-registered as in the first sub-study and 20 image slices (348 µm thickness) were selected from the middle of the core quarter to prevent the possible edge artefacts (from enzymatic treatments and sample preparation). The depth-wise profiles of the 3D
dataset intensities were analysed and compared to the 2D depth-wise profiles obtained from FTIRI.

6.2.2 CEµCT with ioxaglic acid (II)

For the second sub-study, one of the adjacent quarters from each bovine osteochondral core was subjected to CEµCT with ioxaglic acid, commercial name Hexabrix®, as a reference. As Hexabrix® has been developed as a clinical contrast agent, our staining protocol was selected to simulate the in vivo staining of cartilage. In practice there was no fixation of the samples and the ioxaglic acid solution (Hexabrix® 320 mgI/ml in phosphate-buffered saline (PBS), pH 7.4) was diluted to 10%. The sample was immersed in a Hexabrix® solution for 28 hours and subjected to µCT imaging in a sealed container. Due to the relatively fast washout of the Hexabrix®, the samples were fixed to the container walls and cotton ball moistened with PBS were added to the bottom of the container to raise the humidity inside it. The µCT scanning protocol and further image processing were conducted as for the PTA samples in the second sub-study.

6.2.3 CEµCT with CA4+ (III)

For the third sub-study, two human sample sets were subjected to CEµCT with CA4+ as described in section 6.1.3. The first sample set was selected for multiple time point µCT imaging to determine the diffusion of CA4+ in the chondrons and to set optimal CA4+ staining concentration and time for human articular cartilage, and the second sample set with varying OA to determine whether CA4+ accumulation in chondrons affected the low-resolution ex vivo µCT studies.

Three adjacent cores from the first sample set were subjected to CA4+ with concentrations of 3 mgI/ml, 6 mgI/ml and 24 mgI/ml. The samples were imaged with the µCT system (Skyscan 1272, Bruker microCT) at predefined time points of 0, 1, 3, 5, 9, 18, 24, 32, 40, and 48 hours. The scanning parameters were set to be 45 kV, 222 µA, 1200 projections, 3050 ms exposure, averaging two frames/projection, with additional 0.25 mm aluminium filtration, and with 3.2 µm isotropic voxel side length. The projection data were reconstructed using Nrecon software (v.1.6.9.8, Bruker microCT). After reconstruction, Dataviewer software (v. 1.5.1.3., Bruker microCT) was used to sample-wise co-register the datasets of different time points with 6 degrees of freedom. From the co-registered datasets the volume of interest (VOI) of 250 x 250 x Z µm, Z being the sample-wise cartilage
thickness varying from 1200-3100 µm), was selected manually from the centre of the core. The VOIs were subjected to chondron segmentation and further image processing analyses to determine the CA4+ accumulation in chondrons and the optimal CA4+ staining protocol for human articular cartilage.

Based on the results from the first sample set, the sufficient staining time and CA4+ concentration were selected for the second sample set. The cores were immersed in 6 mg/l/ml for 24h. The µCT scans, reconstruction of projection data, and the VOI selection were conducted similarly as for the first sample set. The VOIs were subjected to chondron segmentation and further image processing analyses to determine the relative difference in the X-ray attenuation of chondron areas with high CA4+ accumulation and X-ray attenuation of the rest of the ECM.

6.3 Native-computed tomography of subchondral bone (IV)

For the fourth sub-study, three different native-computed tomography modalities (desktop micro-computed tomography (µCT), synchrotron-based micro-computed tomography (SRCT), and clinical cone-beam computed tomography (CBCT)) were used to image subchondral bone at different resolutions.

6.3.1 Desktop µCT (IV)

For the fourth sub-study, osteochondral samples with diameter 4 mm were imaged with a desktop µCT system (Skyscan 1272, Bruker microCT). The scanning parameters were 50 kV, 200 µA, 1200 projections, 2200 ms exposure, averaging of three frames per projection, additional 0.5 mm aluminium filtration, and isotropic voxel size of 2.75 µm. The projection data were reconstructed using Nrecon software (v. 1.6.10.4, Bruker microCT).

6.3.2 SRCT (IV)

SRCT was conducted at the Canadian Light Source Inc. (Saskatoon, SK, Canada) on the BioMedical Imaging and Therapy (BMIT-ID) 05ID-2 beamline. Human osteochondral samples were scanned at 31 keV, with 0.9 µm voxel side length, and 720 projections were gathered over 180 degrees. Prior to each scan, 10 dark frames and 10 flat-field images were gathered. A custom-made ImageJ plugin was utilised to apply the flat-field correction by first calculating the average dark frame and average flat-field from the measured frames and then calculating the corrected
projection image, \( C \), from the raw projections, \( P \), using the flat-field equation (Nieuwenhove et al., 2015):

\[
C = \frac{P - D}{F - D}
\]

(2)

where \( F \) is the averaged flat-field image, and \( D \) is the averaged dark frame. Subsequently, distortion correction was conducted for the flat-field corrected projections prior to reconstruction. Distortion correction was conducted with the ImageJ plugin (Kaynig, Fischer, Müller, & Buhmann, 2010). The custom-made Barium phantom was scanned to generate the calibration images with a sufficient amount of detail for distortion estimation. After corrections to projections, the projection data was reconstructed using Nrecon software (v. 1.6.10.4, Bruker microCT).

6.3.3 Clinical cone-beam computed tomography (CBCT) (IV)

Clinical CBCT was conducted at Oulu University Hospital (Oulu, Finland) with a CBCT system designed for high-resolution orthopaedic imaging (Planmed Verity®, Planmed Inc., Helsinki, Finland). Human osteochondral cores were imaged with the following scanning parameters: 80 kVp tube voltage, 12 mA tube current, 300 projections, 20 ms pulse time, and 200 \( \mu \)m isotropic voxel size. A sample container was attached with tape to the stage, which was centred close to the gantry’s centre of rotation. The data were reconstructed using the reconstruction software integrated with the equipment with standard data filtering.

6.3.4 Co-registration and volume of interest (VOI) selection

For comparison between SRCT and CBCT, the reconstructed 3D datasets of \( \mu \)CT were co-registered to SRCT and CBCT datasets using Dataviewer software (v. 1.5.4, Bruker microCT). For the SRCT comparison, the rectangular cuboid VOI of 1980x1980x2000\( \mu \)m\(^3\) was selected due to the smaller field of view in SRCT. For the CBCT comparison, a circular VOI of 3000x3000xZ\( \mu \)m\(^3\) (where \( Z \) is the depth from the plate of the trabecular bone) was selected containing only trabecular bone. The datasets were subjected to their corresponding segmentations, morphological analyses and textural analyses.
6.4 Reference methods

6.4.1 Fourier transform infrared imaging (I, II)

Fourier transform infrared imaging (FTIR), was used in the first and second sub-studies to obtain depth-wise distribution of collagen and/or GAG content. Unstained histological sections with thickness 5 µm were imaged using a Hyperion 3000 FTIRI Microscope (Bruker Inc. Billerica, MA, USA).

In the first sub-study (I), unstained histological sections from equines and humans were scanned with 2x2 binning, 5.4x5.4 µm² pixel size, 4 cm⁻¹ spectral resolution, and 32 acquisitions per pixel. The collagen content distribution was evaluated from the area under the Amide I peak (1590-1720 cm⁻¹) of the spectrum.

In the second sub-study (II), unstained bovine histological sections were scanned with 2x2 binning, 5.4x5.4 µm² pixel size, 8 cm⁻¹ spectral resolution, and 16 acquisitions per pixel. The collagen content distribution was evaluated from the area under the Amide I peak (1595-1720 cm⁻¹) and the GAG content distribution was evaluated from the area under the carbohydrate peak (985-1129 cm⁻¹) of the spectrum.

The resulting distribution maps of collagen and/or GAG content were subjected to profile analysis as described in section 5.5.1.

6.4.2 Histological staining and osteoarthritis severity grading (I, II, III, IV)

Conventional histological stains were used as a reference to qualitatively confirm the distribution of articular cartilage constituents (I, II, III), and OA severity using histopathological grading (III, IV). Prior to histological sectioning, the samples were fixed in 10% neutral-buffered formalin for 2 days, then decalcified with EDTA for 14-60 days depending on the time needed for complete decalcification. Next, the samples were embedded in paraffin for the microtome sectioning. Depending on the utilisation of the section, its thickness varied between 3 µm and 5 µm.

In the first sub-study, Masson’s trichrome stain was utilised to qualitatively evaluate the collagen distribution in human articular cartilage. The 5 µm thick histological sections were subjected to Masson’s trichrome stain protocol derived from (Luna, 1968).
In the second sub-study, conventional histology stains for 3µm sectioned slices were used to qualitatively evaluate the effectiveness of enzymatic degradations. Safranin O-stain (Kiviranta, Jurvelin, Tammi, Säämänen, & Helminen, 1985) was used to determine the GAG distribution. The standard picrosirius red-protocol (Schmitz et al., 2010) was modified by adding a purification step to demonstrate collagen fibre patterns and more reliable analysis of collagen distribution. Detailed description of the purification protocol can be found in III. The picrosirius red-stained sections were subjected to polarised light microscopy.

In the third sub-study, adjacent plugs were subjected to conventional histology for visual evaluation between CA4+ and cationic histological stains. Safranin O-stains (Kiviranta et al., 1985) and Toluidine blue-stains were conducted for 3 µm thick sections to visualise the distribution of different cationic stains in articular cartilage.

Furthermore, in the third and fourth sub-studies, 3 µm thick histological sections from human samples were stained with Safranin O-stain (Kiviranta et al., 1985) for OA severity grading according to the histological grading system of the Osteoarthritis Research Society International (Pritzker et al., 2006). The grading was conducted by at least two individual graders and a consensus grade was established in cases of disagreement.

6.4.3 Biochemical analyses (II)

For biochemical analyses in the second sub-study, the quarters of the bovine osteochondral cores were cut in two, from which one half was subjected to uronic acid quantification and the other half proteoglycan structure analysis.

Uronic acid quantification was conducted to provide a reference for GAG depletion due to the enzymatic treatments. The pre-weighted sample quarters were digested in papain (1 mg/ml in 5 mM cysteine and 5 mM EDTA) overnight at 60 °C to solubilise the cartilage tissue. The uronic acid content was determined as described in (Blumenkrantz & Asboe-Hansen, 1973). The results were normalised by the wet weight.

Since the chondroitinase ABC (GAG degradation) and collagenase (collagen degradation) treatments led to similar loss of GAGs from the articular cartilage, the proteoglycan (PG) structures were analysed with agarose gel electrophoresis. The PGs were extracted by immersing the samples in 4 M guanidinium hydrochloride in 50 mM sodium acetate, 10 mM EDTA and 5 mM benzaminidine hydrochloride (pH 5.8) at 4 °C for 72 hours. The amount of the PGs was determined with
dimethylmethylene blue assay (Farndale, Buttle, & Barrett, 1986), and the PGs (5µg GAG) were separated in 1.2% agarose gels as described previously (Qu, Lindeberg, Ylärinne, & Lammi, 2012).

6.4.4 Polarised light microscopy (II)

In the second sub-study, polarised light microscopy was used to measure the retardance caused by the picrosirius red stain in the histological sections to reveal alterations in the collagen content of bovine articular cartilage due to the enzymatic degradations. The sections were imaged using the Abrio polarised light imaging system (CRi Inc., Woburn, MA, USA) mounted on a light microscope (Nikon Diaphot TMD, Nikon Inc., Shinagawa, Tokyo, Japan).

6.5 Image analysis (I, II, III, IV)

6.5.1 Depth-wise profile analyses (I, II)

To compare contrast agent distribution to collagen and/or GAG content distribution in articular cartilage, depth-wise profile analyses were conducted on the CEµCT data and FTIRI reference data in the first and second sub-studies. The first step of the depth-wise profile analyses was detecting the tidemark and cartilage surface, which was implemented by manually selecting the interfaces from the FTIRI data in both sub-studies and the CEµCT data in (I). Interface detection from the CEµCT data was automated for the second sub-study, because the analyses were done in 3D. The automated interface detection was based on the strong image gradient at the surface and at the tidemark of the articular cartilage generated by PTA accumulation. After detection, the interfaces were straightened by normalising each column of the image matrix followed by linear interpolation. In the first sub-study, the normalisation was set so that zero depth represented the surface of the articular cartilage, the tidemark was set to 0.72, and the calcified cartilage-bone interface was set to 1. In the second sub-study, depth from surface, which was set to zero, to tidemark was resampled to 100 data points. After the normalisation, the horizontal averages were calculated, resulting in a 2D depth-wise profile. The 3D depth-wise profile of the CEµCT data in the second sub-study was generated by averaging the 2D depth-wise profiles from the 20 cross-sectional slices of the VOI.
In the first sub-study (I), the image intensity values in the profiles were furthermore normalised to provide profile comparison between the FTIRI and CEµCT profiles.

In the second sub-study (II), the depth-wise profiles from the CEµCT and FTIRI were evaluated point-by-point with a non-parametric Wilcoxon signed-rank test. Furthermore, the point-by-point relative difference between enzymatically treated sample \( x_{\text{Enz}} \) and control sample \( x_{\text{con}} \) for each sample were calculated using equation 3:

\[
\text{Relative difference (\%)} = 100 \times \frac{x_{\text{Enz}} - x_{\text{con}}}{x_{\text{con}}}
\]

\[ (3) \]

### 6.5.2 Analysing CA4+ diffusion to chondrons (III)

To study the CA4+ diffusion in chondrons and the rest of the ECM separately, CA4+ accumulation after 48 hours of staining was used for chondron and interterritorial matrix (ITM) segmentation. Segmentation and prefiltering were conducted using CTAn software (v. 1.15.4.0, Bruker microCT). Prior to segmentation, noise was filtered with two consecutive filters: uniform and unsharp mask filters. Segmentation was conducted by applying a multilevel OTSU threshold (Otsu, 1979) in 3D and selecting the level with the highest grey-level values as a mask for the chondron volume. The mask for the ITM volume was selected to be the rest of the VOI. Applying these masks, generated from 48-hour time point data, to the other time points provided investigation of intensity alterations caused by contrast agent diffusion as a function of time of chondron volume and ITM volume separately.

The datasets were grouped by the CA4+ concentration. The grey-level values, corresponding to the X-ray attenuation of CA4+ stained cartilage, were averaged volume-wise and group-wise, and the group-wise standard deviation was calculated. Thus, the mean grey-level values were calculated for ITM volume, chondron volume and the unsegmented volume for each time point. The mean grey-level values were normalised inside the groups before fitting equation 4, which was derived from previous CA4+ studies (Bansal et al., 2011; Mashiatulla et al., 2017):

\[
A = A_{\text{max}} - K \times e^{-t/\tau},
\]

\[ (4) \]

where \( A \) is the normalised X-ray attenuation, \( t \) is time, \( A_{\text{max}} \) is the maximum X-ray attenuation, \( K \) is the fit-dependent variable, and \( \tau \) is the time constant. Based on equation 4, the sufficient staining times were calculated for each fit by defining the
sufficient staining time to be reached when the ratio of $A_{\text{max}}$ and $A$ reached 0.98. MATLAB software (v. 2015a, MathWorks Inc., Natick, MA, USA) was used for the fitting.

To determine how CA4+ accumulation to chondrons would affect the low-resolution *ex vivo* µCT studies analysing the GAG content in full tissue volume, a sample set imaged with the optimised CA4+ protocol was subjected to similar analysis steps as described earlier. The VOIs were prefiltered with uniform and unsharp mask filters and segmented with the multilevel OTSU method (Otsu, 1979). The averaged X-ray attenuation of the segmented ITM volume and unsegmented volume were calculated. The relative difference (see equation 3) was calculated between the X-ray attenuation of ITM volume and unsegmented (full tissue) volume.

### 6.5.3 Morphological analysis of subchondral bone (IV)

As a reference for texture analyses, relevant metrics from the conventional bone morphometrics (Bouxsein et al., 2010) were calculated using CTAn (v. 1.17.7.2., Bruker microCT) from their respective VOIs described in section 6.3.4.. To investigate how the grey-level co-occurrence matrix (GLCM) contrast of µCT data corresponds to the osteocyte lacunae cellularity, the ratio between the osteocyte lacunae volume and bone volume was calculated from the SRCT data. The bone was pre-masked by global thresholding of the µCT data, excluding the trabecular and vessel cavities. As the µCT and SRCT datasets were co-registered, the same bone mask was applicable to the SRCT. The osteocyte lacunae volume was segmented inside the mask by applying the global threshold.

When investigating how the textural parameters from CBCT would correlate with the morphological parameters obtained from µCT imaged trabecular bone, traditional morphological parameters were calculated using CTAn (v. 1.17.7.2., Bruker microCT): bone volume fraction (BV/TV), trabecular number (Tb.N.), trabecular thickness (Tb.Th.), trabecular separation (Tb.Sp.), and local bone surface complexity (fractal dimension, FD).

BV/TV is a measure of the total bone volume in the region of interest (Parfitt et al., 1987). It is defined as a ratio between the bone volume ($BV$) and the total volume of interest ($TV$) as described in equation 5:

$$BV/TV (\%) = 100 \times \frac{BV}{TV}. \quad (5)$$
Tb.Th. and Tb.Sp. are defined from the 3D volume by the sphere-fitting method, in which in the case of Tb.Th. the spheres are fitted in the segmented bone and in Tb.Sp. the fitting is conducted on the trabecular holes (Hildebrand & Rüegsegger, 1997). A diameter value of the maximum size of sphere which can fit inside the structure or a hole was calculated for each voxel. The mean of the sphere diameters was calculated after fitting, resulting in the Tb.Sp. and Tb.Th. values. The Tb.N. describes the amount of trabeculae per unit length. It is related to the Tb.Th. and Tb.Sp. calculations as the Tb.N. is calculated as described in equation 6:

\[
Tb.N. = \frac{1}{(\text{Tb.Th.} + \text{Tb.Sp.})}
\]  

(6)

FD describes the surface complexity of an object, quantifying how the object's surface fills the space. Fractal dimension in this study is calculated using the box-counting method (Chappard et al., 2001). Briefly described, the volume is divided into smaller cubes and the number of cubes containing the object surface is counted. This calculation is repeated with a range of cube sizes from 2-100 voxels, resulting in a log-log plot containing the number of cubes as a function of the cube length. The fractal dimension is obtained from the log-log regression.

6.5.4 Texture and histogram analysis of subchondral bone (IV)

Histogram and texture analysis were conducted from the µCT and CBCT images and compared to the morphometrics obtained from SCT and µCT, respectively. MATLAB (v. 2017b, MathWorks) was used to calculate the texture and histogram parameters. The histogram parameters calculated were mean, standard deviation, skewness, kurtosis and image entropy (see equation 13). The texture analysis conducted in the fourth sub-study were based on the second-degree statistics from the grey-level co-occurrence matrix (GLCM).

Grey-level co-occurrence matrix (GLCM) based texture analysis was described first by Haralick et al. (Haralick et al., 1973; Haralick, 1979). It extracts second-order statistical features from grey-level images. The GLCM consists of elements \( P(i,j) \), which represent the number of grey-level co-occurrences of each \( i \) and \( j \) pixel/voxel pair from the original grey-level image: \( i \) and \( j \) are defined based
on the displacement $d$ and displacement angle $\theta$ (or in a 3D matrix $[0, \varphi]$ for 3D coordinate angles). The GLCM size is restricted by the number of quantised grey-levels $N_g$, resulting in $N_g \times N_g$ sized GLCM (see Figure 3).

![Fig. 3. Construction of the grey-level co-occurrence matrix (GLCM). The greyscale image in the example is 4-by-4 with four grey-level values (0-3) and it results in 4-by-4 GLCM.](image)

The second-order statistical probability values $p(i,j)$ are calculated with equation 7, to normalise the GLCM:

$$p(i,j) = \frac{P(i,j)}{\sum_{i=0}^{N_g-1} \sum_{j=0}^{N_g-1} P(i,j)} \quad (7)$$

In 3D, as in the case of the fourth sub-study, there are 13 unique displacement angles. The displacements and unique displacement angles are presented in Table 1.

The parameters $d$ and $N_g$ affect the texture parameters calculated from the GLCM (Clausi, 2002). In the 8-bit grey-level image, $N_g$ can obtain values up to 256. The reduction of $N_g$ can decrease noise at the expense of information, but the calculation time decreases. Previously it has been suggested that sufficient accuracy of the textural parameters can be obtained with $N_g$ values of 24 or higher (Clausi, 2002).

In the fourth sub-study, we applied the GLCM to 3D data, thus the GLCM was calculated in all the 13 unique angles and summed to provide rotationally invariant texture features. As we studied the GLCM texture parameters’ association with the sub-resolution morphometrics, displacement $d$ was selected to be 1. The $N_g$ was selected to be 24 for the µCT to obtain feasible calculation times while maintaining
sufficient accuracy of the textural analysis. The partial volume effect is stronger for the CBCT data because the trabecular bone features are multiple times smaller than the voxel size in CBCT. Furthermore, the data size was more feasible in CBCT, thus we selected $N_g$ to be 256 for the CBCT data analysis.

**Table 1.** The 13 unique displacements utilised in 3D GLCM

<table>
<thead>
<tr>
<th>Direction $(\theta, \phi)$</th>
<th>Displacement in 3D matrix $d(x, y, z)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0°,45°)</td>
<td>(1,0,1)</td>
</tr>
<tr>
<td>(0°,90°)</td>
<td>(1,0,0)</td>
</tr>
<tr>
<td>(0°,135°)</td>
<td>(1,0,-1)</td>
</tr>
<tr>
<td>(45°,45°)</td>
<td>(1,1,1)</td>
</tr>
<tr>
<td>(45°,90°)</td>
<td>(1,1,0)</td>
</tr>
<tr>
<td>(45°,135°)</td>
<td>(1,1,-1)</td>
</tr>
<tr>
<td>(90°,45°)</td>
<td>(0,1,1)</td>
</tr>
<tr>
<td>(90°,90°)</td>
<td>(0,1,0)</td>
</tr>
<tr>
<td>(90°,135°)</td>
<td>(0,1,-1)</td>
</tr>
<tr>
<td>(135°,45°)</td>
<td>(-1,1,1)</td>
</tr>
<tr>
<td>(135°,90°)</td>
<td>(-1,1,0)</td>
</tr>
<tr>
<td>(135°,135°)</td>
<td>(-1,1,0)</td>
</tr>
<tr>
<td>(-90°)</td>
<td>(0,0,1)</td>
</tr>
</tbody>
</table>

From the generated GLCMs, the textural parameters which were often reported in literature were calculated. Of the prominently relevant parameters, some describe similar characteristics and thus in the fourth sub-study, parameters with minimal overlapping in the description were selected. The calculated parameters (Inverse Difference Moment (IDM), Angular Second Moment (ASM), Contrast, Variance, Correlation, Entropy, and Cluster Shade) are presented below.

IDM, also known as homogeneity, is a measure of the local homogeneity. The denominator (see equation 8) emphasises pixel/voxel pairs with close grey-level values. This results in a relatively higher IDM value in homogenous images. IDM has a relationship to the GLCM contrast parameter, as with low contrast the IDM tends to be high. IDM is defined from GLCM using equation 8:

$$IDM = \frac{\sum_{i=1}^{N_g} \sum_{j=1}^{N_g} p(i,j) \left( \frac{1}{1 + (i-j)^2} \right)}{\sum_{i=1}^{N_g} \sum_{j=1}^{N_g} p(i,j)}$$

(8)
ASM, also known as energy or uniformity, describes the overall homogeneity of the image. With increasing textural uniformity or homogeneity, the ASM also increases because in a homogenous image the GLCM results in few high \( p(i,j) \) values resulting in a high sum of squares. ASM is calculated using equation 9:

\[
\sum_{i=1}^{N_g} \sum_{j=1}^{N_g} p(i,j)^2.
\]  

Contrast is a measure of local grey-level variation in the image. High values of contrast can indicate the presence of a large local gradient in the image \((i.e.\ edges)\). Contrast is calculated using equation 10:

\[
\sum_{n=0}^{N_g-1} (i - j)^2 \left\{ \sum_{i=1}^{N_g} \sum_{j=1}^{N_g} p(i,j) \right\}.
\]  

Variance, also known as sum of squares, describes the global variance of the image. Unlike contrast, variance does not measure spatial frequency. The values dispersing from the mean of \( p(i,j) \) \((\mu \text{ in equation 11})\) are given high weights in the variance equation:

\[
\sum_{i=1}^{N_g} \sum_{j=1}^{N_g} (i - \mu)^2 p(i,j).
\]  

Correlation describes the linear dependency between neighbouring pixels/voxels. Thus high local grey-level dependency \((i.e.\ similar\ grey-level\ regions\ in\ the\ image)\) results in high correlation values. The equation for calculating the correlation utilises mean \((\mu_x, \mu_y)\) and standard deviations \((\sigma_x, \sigma_y)\) of the row and column sums of the GLCM:

\[
\sum_{i=1}^{N_g} \sum_{j=1}^{N_g} \frac{(i-j)(p(i,j)-\mu_x \mu_y)}{\sigma_x \sigma_y}.
\]  

Entropy is a measure of the randomness of the texture or intensity distribution. It has an approximately inverted relationship to uniformity. It is to be noted that image entropy can be calculated from the image histogram using the same equation. The difference is that GLCM entropy describes randomness in the texture as image
entropy describes randomness in the grey-level values. Entropy is calculated using equation 13:

\[-\sum_{i=1}^{N_x} \sum_{j=1}^{N_y} p(i,j) \log(p(i,j)). \quad (13)\]

A cluster shade is a measure of asymmetry. It describes the skewness of the GLCM. It is calculated using equation 14:

\[\sum_{i=1}^{N_x} \sum_{j=1}^{N_y} (i \cdot j - \mu_x \cdot \mu_y)^3 \cdot p(i,j). \quad (14)\]

6.6 Statistical analyses (I-IV)

The statistical analyses in this thesis were conducted using IBM SPSS Statistics software (in II v.21, in III v.24, in IV v. 25; International Business Machines Corporation, Armonk, NY, USA), except the first sub-study’s Pearson’s correlations and Bland-Altman analysis, and third sub-study’s goodness-of-fit analysis, which were conducted using MATLAB software (in I v.2013a, in III v. 2015a; MathWorks Inc., Nantick, MA, USA).

In first sub-study, normalised depth-profiles of equine cartilage acquired by FTIRI and both CEμCT methods (PTA and PMA) were compared with Pearson’s correlation and Bland-Altman analyses at each time point \((n=12\) per time point). The Pearson’s correlation coefficient was also calculated for the second part of the study with human cartilage \((n=4)\) to compare the normalised FTIRI and μCT depth-profiles.

In the second sub-study, depth-profiles were acquired from FTIRI and two different CEμCT methods (PTA and Hexabrix®). The depth-profiles of samples with enzymatic degradation with collagenase or chondroitinase ABC were compared with depth-profiles of untreated samples \((n=9\) per group). A non-parametric two-tailed Wilcoxon signed-rank test was conducted at each depth, resulting in 100 consecutive statistical comparisons per enzymatic treatment vs. the untreated group comparison. The biological variation within the samples were estimated by calculating the coefficient of variation of pixel/voxel value for the samples from the untreated group. Furthermore, a 2-tailed paired t-test was used for testing the differences in agarose gel profiles and uronic acid content between the enzymatically treated and untreated sample groups.
In the third sub-study, the goodness-of-fit was quantified by calculating the coefficient of determination ($R^2$) to evaluate how well the fit corresponded to the measured diffusion data. Furthermore, in the third sub-study, the CA4$^+$ intensity in chondron volume, ITM and the full sample was correlated to osteoarthritis severity (OARSI grade) using a Spearman correlation.

To evaluate the association between the morphological parameters and histogram/GLCM parameters, Pearson’s correlation coefficients were calculated in the fourth sub-study. The Spearman’s correlation coefficient was calculated to estimate the GLCM/histogram and morphological parameters’ association with the OARSI grade, because the OARSI grade is ordinal. In addition to the individual parameter comparisons, stepwise linear regression was used to find the most optimal estimate of morphological parameters from the linear regression of multiple GLCM/histogram parameters. The variance inflation factor (VIF) was calculated to evaluate the collinearity in the models. Parameters with VIF>5 were excluded from the models. In addition, collinearity was checked qualitatively by evaluating the P-P-plot of regression standardised residuals.

In the fourth sub-study, the effect of core location was conducted qualitatively by comparing the individual parameter correlations and more quantitatively by adding the location information to the linear regression models of multiple GLCM/histogram parameters. The core location information was sub-grouped based on the origin of the core (TKA patients and cadavers), compartmental location (medial and tibial plateau, femurs excluded), and areal location (central, anterior, posterior, and distal tibial plateaus, femurs excluded). The number of cores per subgroup, exclusions and areal locations are presented in Figure 2.
7 Results

7.1 CEµCT of collagen content of articular cartilage (I, II)

In the first sub-study, both the PMA and PTA distributions in equine cartilage had a high correlation (Pearson’s $r > 0.90$) to collagen content measured by FTIRI in most of the samples at least one or more time points during the staining immersion (see Table 2). An increase of X-ray intensity, due to contrast agent accumulation, in the surface of the articular cartilage and tidemark was observed with both contrast agents after 36 hours. Prolonging the staining over 36 hours with PMA, and over 54 hours with PTA, resulted in excessive stain accumulating on the surface of articular cartilage and in the tidemark.

Table 2. Means and standard deviations (SD) of Pearson’s correlation coefficients from FTIRI vs. CEµCT comparison of depth-wise image intensities. The highest correlation coefficients per contrast agent are bolded. Overall, the highest correlation was obtained with 36 and 54 hour immersion in PTA. The Pearson’s correlation coefficients for each sample are presented in (I).

<table>
<thead>
<tr>
<th>Immersion time in hours</th>
<th>PTA (n=12) Pearson’s r mean ± SD</th>
<th>PMA (n=12) Pearson’s r mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>0.92 ± 0.10</td>
<td>0.93 ± 0.07</td>
</tr>
<tr>
<td>36</td>
<td>0.96 ± 0.03</td>
<td>0.90 ± 0.14</td>
</tr>
<tr>
<td>54</td>
<td>0.96 ± 0.03</td>
<td>0.84 ± 0.18</td>
</tr>
<tr>
<td>72</td>
<td>0.95 ± 0.03</td>
<td>0.81 ± 0.19</td>
</tr>
<tr>
<td>90</td>
<td>0.93 ± 0.04</td>
<td>0.73 ± 0.27</td>
</tr>
<tr>
<td>180</td>
<td>0.92 ± 0.04</td>
<td>0.63 ± 0.29</td>
</tr>
<tr>
<td>270</td>
<td>0.90 ± 0.05</td>
<td>0.54 ± 0.30</td>
</tr>
</tbody>
</table>

PTA staining for 36 and 54 hours gave highest correlation with collagen content, as the Pearson’s correlation coefficients, averaged from the 12 samples, were $r = 0.96 ± 0.03$ in both cases. Based on the Bland-Altman analysis, the best correspondence with FTIRI-detected collagen content was also with PTA at 36-hour and 54-hour time points. With those time points, 95% of the µCT image intensity data points deviated from the corresponding FTIRI data points by less than 18.1%. PMA yielded the highest correlations to FTIRI at 18-hour staining ($r = 0.93 ± 0.07$; and 95% of data points deviated <25.1 %); however, PTA gave higher correlations with both comparison methods, and thus PTA with 36-hour staining time was selected
as the optimal protocol for staining articular cartilage. This optimised CEµCT protocol qualitatively showed similar collagen content distribution to Masson’s trichrome histological stain and FTIRI. Furthermore, the PTA stain allowed 3D visualisation of the collagen distribution within articular cartilage. Quantitatively, the Pearson’s correlation coefficients between PTA distribution and collagen content in human cartilage (n=4) were 0.86, 0.61, 0.83 and 0.96.

In the second sub-study, the specificity of the PTA for collagen content and distribution was studied more extensively. Qualitatively, the PTA distribution in bovine cartilage showed similar depth-wise distribution with collagen-specific histological stain, picrosirius red, with and without enzymatic treatments. Qualitative comparison to other anionic contrast agents for CEµCT, Hexabrix®, as well as the GAG-specific histological stain, Safranin O, did not provide similar distributions with PTA (Fig. 2).

The collagenase-degraded group showed a statistically significant decrease (p<0.05) of 14-40% in X-ray intensity at a 0-13% tissue depth, and a 9-10% decrease in the X-ray intensity at a 48-63% tissue depth of the PTA-stained articular cartilage when compared to the untreated group (Fig. 3). This corresponded spatially to the collagen content decrease observed by the FTIRI analyses of the comparison between collagenase treated and untreated groups. Collagenase degradation also showed statistically significant PG depletion at 0-77% tissue depth, but no significant increase in X-ray attenuation of CEµCT with Hexabrix®.

The chondroitinase ABC-treated samples had significantly (p<0.05) increased X-ray attenuation of PTA-stained articular cartilage at tissue depths of 13-39% compared to untreated samples (Fig. 3), even though statistically significant changes were not observed in the collagen content measured by FTIRI. Statistically significant (p<0.05) decrease of PGs was observed with FTIRI at tissue depths of 0-73% and a statistically significant (p<0.05) increase in X-ray attenuation of Hexabrix® stained articular cartilage was observed at tissue depths of 22-48%.

The uronic acid analysis confirmed a statistically significant loss of GAGs with both enzymatic treatments (chondroitinase ABC p=0.001, and collagenase p=0.019) when compared to the untreated group. Furthermore, the PG structure analysis with agarose gel electrophoresis showed an increase presence of diffuse higher-mobility PGs and the average profiles of the enzymatically treated samples revealed significant (p<0.05) differences in staining patterns with both enzymatic treatments.

Biological sample-to-sample variation was also measured in the first sub-study. It was 8-16% in CEµCT with PTA, 12-15% in CEµCT with Hexabrix®, 14-26% in collagen content, and 16-22% in PG content measured by FTIRI.
Fig. 4. Comparison of histological stains with 2D slices from CEµCT with PTA and Hexabrix®. Both enzymatic treatments showed depletion of PGs which resulted in similar depth-wise distribution of the labelling agent between histology (Safranin O-stain) and CEµCT (Hexabrix®). PTA distribution did not seem to modify with PG depletion but rather corresponded with collagen depletion by the collagenase treatment, which was also visible with the picrosirius-stained histological slice. The black arrows indicate the cartilage surface and bone-cartilage interface, the red arrows indicate the “front” of the enzymatic degradation if distinguishable. The scale bars on the right corner of each sample equal 500 µm. Figure obtained from the second sub-study (II).
Fig. 5. A-D: Relative differences between enzymatically treated and untreated sample groups. Depth-wise analyses show relative differences of image intensities with FTIRI measured collagen (B) and PG (D) content and relative differences of volume intensities of CEµCT with PTA (A) and Hexabrix® (C). The statistical significance is represented underneath each graph as the statistical comparison between groups was made point-by-point depth-wise. E: The uronic acid analysis confirmed the statistically significant depletion of GAGs with both enzymatic treatments when compared to the untreated group. Figure obtained from the second sub-study (II).

7.2 CEµCT with CA4+ of human articular cartilage (III)

In the third sub-study, the CA4+ accumulation in chondrons was observed with all tested CA4+ concentrations. Qualitatively, the depth-wise distribution of CA4+ in human articular cartilage corresponded to the depth-wise distribution of cationic histological stains of Toluidine blue and Safranin O from the adjacent osteochondral cores. Both cationic stains also accumulated more strongly in the chondrons, when compared to the rest of the ECM. Furthermore, Toluidine blue also seemed to accumulate outside the chondrons partially in the territorial matrix. This area corresponded roughly with the size of the accumulation area in the CA4+ stained articular cartilage (Fig. 4).
Fig. 6. A: Visual comparison between cationic histological stain distributions (Toluidine blue and Safranin O) and different CA4+ concentration distributions in CEpCT after 48 hours immersion in the contrast agent. With all CA4+ concentrations CA4+ seems to accumulate in chondrons. B: Magnification of the yellow areas in A. Similarly as in Toluidine blue, the cationic CA4+ accumulation goes outside the chondron to the territorial matrix. Figure obtained from third sub-study (III).

Quantitatively, strong correlations were obtained ($R^2>0.97$) from fitting equation 4 to the data points of the mean X-ray attenuation values from the chondron, ITM and full tissue volume as a function of time (Fig. 5 and Table 3). The time constant and sufficient staining times, calculated from equation 4, were similar in the ITM and full tissue volume but much lower when compared to the time constants and staining times obtained from the chondron volume (Table 3). CA4+ concentrations of 3 mg/l/ml and 6mg/ml gave out similar the time constants and sufficient staining times for all volumes, while with 24 mg/l/ml the staining times and time constants were smaller. Based on the qualitative evaluation, a CA4+ concentration of 6 mg/l/ml was selected due to its good contrast between chondrons and ITM for evaluating the effect of CA4+ accumulation in lower-resolution µCT scans. The results indicate that the sufficient staining time for CA4+ to accumulate in chondrons was ~15 hours with 6 mg/l/ml concentration, thus we selected 24-hour staining time to ensure that the staining is complete even with samples with varying OA severity.
Fig. 7. Normalised and averaged X-ray intensities from chondron, ITM and unsegmented volumes with the profiles fitted to the data points by equation 4. Data points are averaged for each CA4+ concentration and standard deviations inside the groups for each time point are presented in the graph. With all concentrations, the ITM and unsegmented volume gave similar results. Figure obtained from third sub-study (III).
Table 3. The time constants (τ), goodness of fits (R^2), and sufficient staining times for chondron, ITM and unsegmented volume with different CA4+ concentrations. Time constants and sufficient staining times are based on the fitted profiles. The sufficient staining times reported here are determined by when the X-ray attenuation of CA4+ has reached 98% of the maximum (see equation 4) inside the respective volume.

<table>
<thead>
<tr>
<th>CA4+ concentration</th>
<th>Volume</th>
<th>τ</th>
<th>R^2</th>
<th>Sufficient staining time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mg/l/ml</td>
<td>Unsegmented</td>
<td>1.678</td>
<td>0.985</td>
<td>10.45</td>
</tr>
<tr>
<td></td>
<td>ITM</td>
<td>1.617</td>
<td>0.981</td>
<td>10.07</td>
</tr>
<tr>
<td></td>
<td>Chondron</td>
<td>2.487</td>
<td>0.991</td>
<td>15.38</td>
</tr>
<tr>
<td>6 mg/l/ml</td>
<td>Unsegmented</td>
<td>1.800</td>
<td>0.993</td>
<td>11.16</td>
</tr>
<tr>
<td></td>
<td>ITM</td>
<td>1.749</td>
<td>0.994</td>
<td>10.85</td>
</tr>
<tr>
<td></td>
<td>Chondrons</td>
<td>2.486</td>
<td>0.979</td>
<td>15.38</td>
</tr>
<tr>
<td>24 mg/l/ml</td>
<td>Whole AC</td>
<td>1.216</td>
<td>0.985</td>
<td>7.55</td>
</tr>
<tr>
<td></td>
<td>ITM</td>
<td>1.203</td>
<td>0.986</td>
<td>7.47</td>
</tr>
<tr>
<td></td>
<td>Chondrons</td>
<td>1.374</td>
<td>0.971</td>
<td>8.52</td>
</tr>
</tbody>
</table>

Utilising this optimised protocol for a larger sample set (n=14) with varying OARSI grades (1.0-4.5) revealed that the relative difference of averaged X-ray intensity between the ITM and full tissue volume was less than 4%. No correlation between the OARSI grading and the averaged X-ray intensities of CA4+ stained samples at any segmented volume was found (Spearman’s ρ: 0.070 at full tissue volume, 0.070 at ITM volume, and -0.047 at chondron volume).

7.3 Bone structure estimation with CT methods (IV)

Subchondral bone osteocyte cellularity measured with SRCT had the strongest, and statistically significant, correlation with the GLCM (r = -0.472) of all the GLCM and histogram parameters calculated from the μCT data (see Table 4). A stepwise linear regression model consisting of a combination of GLCM correlation and GLCM cluster shade parameters resulted in stronger, but moderate correlation than any of the individual parameters (r = 0.519). Comparison of individual parameter correlations revealed that the associations were not robust to the location of the cores (presented in Supplementary Tables 2-4 in IV). However, the combined model of GLCM correlation and GLCM cluster shade parameters was not affected when sub-grouping information for TKA/cadaver groups (adjusted R^2 change: -0.01) and compartmental locations (adjusted R^2 change: -0.02). Still, the areal locations did affect the model (adjusted R^2 change: 0.042).
Table 4. Correlations between SRCT morphometry, µCT GLCM and histogram parameters, and OARSI grade. Asterisks (*) indicate the statistical significance of the correlations (**: \( p<0.001 \), ***: \( p<0.01 \), *: \( p<0.05 \)).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SRCT morphometrics</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cellularity (Pearson’s ( r ))</td>
<td>OARSI (Spearman’s ( \rho ))</td>
</tr>
<tr>
<td>Histogram parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.439**</td>
<td>0.598**</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.294*</td>
<td>0.302*</td>
</tr>
<tr>
<td>Skewness</td>
<td>-0.097</td>
<td>0.111</td>
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<tr>
<td>Kurtosis</td>
<td>-0.135</td>
<td>0.067</td>
</tr>
<tr>
<td>Image Entropy</td>
<td>0.357**</td>
<td>0.564**</td>
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<tr>
<td>GLCM texture parameters</td>
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<td></td>
</tr>
<tr>
<td>Contrast</td>
<td>0.468**</td>
<td>0.554**</td>
</tr>
<tr>
<td>Correlation</td>
<td>-0.472**</td>
<td>-0.589**</td>
</tr>
<tr>
<td>Cluster Shade</td>
<td>-0.215</td>
<td>-0.424**</td>
</tr>
<tr>
<td>ASM</td>
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<td>-0.622**</td>
</tr>
<tr>
<td>Entropy</td>
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<td>0.625**</td>
</tr>
<tr>
<td>IDM</td>
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<td>-0.619**</td>
</tr>
<tr>
<td>Variance</td>
<td>0.365**</td>
<td>0.414**</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OARSI (Spearman’s ( \rho ))</td>
<td>0.331*</td>
<td></td>
</tr>
</tbody>
</table>

All the trabecular bone morphometrics measured with µCT had statistically significant correlation with several GLCM and histogram parameters calculated from the CBCT data (see Table 5). BV/TV had strongest, and statistically significant, correlation to the histogram mean \( (r = 0.907) \). Similarly, the strongest correlations of histogram/GLCM parameters to other trabecular bone morphometrics were Tb.N. with histogram mean \( (r = 0.834) \), Tb.Th. with histogram standard deviation \( (r = 0.676) \), Tb.Sp. with GLCM variance \( (r = -0.803) \), and FD with GLCM correlation \( (r = 0.759) \). As BV/TV is co-dependent with the other morphometrics, a linear stepwise regression model was generated to estimate BV/TV only. The model consisting of the histogram mean and GLCM IDM had a stronger correlation to BV/TV \( (r = 0.932) \) than any of the individual parameter correlations. Correlations to BV/TV were robust to extraction location of the cores with the individual correlation (the strong correlation of the histogram mean remained with all sub-groups, see Supplementary Tables 5-7 in IV) and with the combined model of histogram mean and GLCM IDM (TKA/Cadaver grouping: 62
adjusted $R^2$ change -0.01; compartmental grouping: adjusted $R^2$ change -0.002; areal grouping: adjusted $R^2$ change -0.001).

The OARSI had statistically significant association with all the measured morphometrics (osteocyte cellularity: $r = 0.331$, BV/TV: $r = 0.584$, Tb.Th.: $r = 0.573$, Tb.Sp.: $r = -0.461$, Tb.N.: $r = 0.479$, and FD: $r = 0.388$). Furthermore, the GLCM and histogram parameters calculated from the µCT and CBCT had statistically significant correlations with OARSI (see Tables 4 and 5). The strongest correlation with OARSI of the µCT GLCM/histogram parameters was with GLCM entropy ($r = 0.625$), and of the CBCT GLCM/histogram parameters was with the histogram standard deviation ($r = 0.663$).

Table 5. Correlations between µCT morphometry, CBCT GLCM and histogram parameters, and OARSI grade (n=53). Asterisks (*) indicate for the statistical significance of the correlations (**: p<0.001, *: p<0.01, *: p<0.05).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>BV/TV (Pearson’s $r$)</th>
<th>Tb.Th. (Pearson’s $r$)</th>
<th>Tb.Sp. (Pearson’s $r$)</th>
<th>Tb.N. (Pearson’s $r$)</th>
<th>FD (Pearson’s $r$)</th>
<th>OARSI (Spearman’s $\rho$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histogram parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.907**</td>
<td>0.606**</td>
<td>-0.792**</td>
<td>0.834**</td>
<td>0.691**</td>
<td>0.612**</td>
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<tr>
<td>Standard Deviation</td>
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<td>-0.757**</td>
<td>0.795**</td>
<td>0.672**</td>
<td>0.663**</td>
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<td>0.083</td>
<td>-0.052</td>
<td>-0.097</td>
<td>-0.054</td>
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<tr>
<td>Kurtosis</td>
<td>0.154</td>
<td>0.265</td>
<td>0.026</td>
<td>0.026</td>
<td>-0.026</td>
<td>-0.052</td>
</tr>
<tr>
<td>Image Entropy</td>
<td>-0.129</td>
<td>0.165</td>
<td>0.278*</td>
<td>-0.215</td>
<td>-0.063</td>
<td>-0.066</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contrast</td>
<td>-0.059</td>
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<td>-0.104</td>
<td>-0.052</td>
<td>-0.185</td>
<td>-0.131</td>
</tr>
<tr>
<td>Correlation</td>
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<td>0.522*</td>
<td>-0.751**</td>
<td>0.776**</td>
<td>0.759**</td>
<td>0.488**</td>
</tr>
<tr>
<td>Cluster Shade</td>
<td>0.891</td>
<td>0.600</td>
<td>-0.803</td>
<td>0.825</td>
<td>0.718</td>
<td>0.612</td>
</tr>
<tr>
<td>ASM</td>
<td>-0.346</td>
<td>-0.079</td>
<td>0.592**</td>
<td>-0.404**</td>
<td>-0.216</td>
<td>-0.280**</td>
</tr>
<tr>
<td>Entropy</td>
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<td>-0.628</td>
<td>0.454</td>
<td>0.264</td>
<td>0.307</td>
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<tr>
<td>IDM</td>
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<td>-0.035</td>
<td>0.571**</td>
<td>-0.382**</td>
<td>-0.178</td>
<td>-0.285**</td>
</tr>
<tr>
<td>Variance</td>
<td>0.825**</td>
<td>0.436</td>
<td>-0.820**</td>
<td>0.804**</td>
<td>0.640**</td>
<td>0.480**</td>
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<tr>
<td>Histology OARSI</td>
<td>0.584**</td>
<td>0.573**</td>
<td>-0.461**</td>
<td>0.479**</td>
<td>0.388**</td>
<td>0.388**</td>
</tr>
</tbody>
</table>

(Spearman’s $\rho$)
8 Discussion

8.1 PTA distribution is associated with collagen distribution (I, II)

In the first sub-study, the association of PTA and PMA distributions with articular cartilage collagen distribution were investigated in equine and human tissue. At the optimised immersion times, both contrast agents correlated well (Pearson’s $r > 0.90$) with the collagen content of the equine cartilage measured with FTIRI. However, PTA yielded higher correlations (see Table 2) and was, therefore, selected for further analyses.

The results from the second sub-study supported the specificity of PTA to collagen content, as the collagenase-degraded bovine cartilage showed visually (see Fig. 2) and spatially (see Fig. 3) a similar decrease in CEµCT intensity as in the reference methods for collagen content determination, i.e. FTIRI and conventional histology.

The PTA stain is based on lowering the pH of the stainable environment and thus rendering the collagen net charge positive, which will then attract the highly anionic PTA molecules to the collagen network through electrochemical forces. However, the FCD of cartilage is negative, due to the anionic GAG content, and as GAGs still have minor but existent negative charge even at the low pH generated by PTA, the repulsive forces of FCD inhibit PTA diffusion to cartilage. The results from the second sub-study demonstrated that GAG content, indeed, has a minor effect on PTA distribution. This can be seen spatially when comparing untreated and chondroitinase ABC-treated samples in a CEµCT setting. Furthermore, a similar increase in X-ray attenuation with PTA and GAG-specific anionic contrast agent, Hexabrix®, in neutral pH (~7), can be observed.

The CEµCT with PTA shows potential as a 3D histological tool for OA-related features as demonstrated in the first sub-study. The PTA distribution correlates well with the collagen content of human articular cartilage as with the animal tissues. Furthermore, CEµCT with PTA provides possibilities for more quantitative analyses of the cartilage surface and ECM integrity due to the histological-quality visualisation of the articular cartilage in 3D.
8.2 CA4+ accumulates in chondrons (III)

The results from the third sub-study are the first to report the accumulation of cationic contrast agent in the chondrons in human articular cartilage. Our results indicate that CA4+ accumulates in the chondrons and partially in the territorial matrix with all tested CA4+ concentrations. Furthermore, this corresponded with the accumulation patterns of cationic histological stains in and around the chondrons (see Fig. 4). This phenomenon can be seen constantly with cationic histological stains, explained not only by the GAG content but also the negative phosphate groups present on the nucleic acids. However, histological staining is conducted on thin tissue sections, and therefore, CA4+ staining of intact tissue volumes might be primarily caused by the GAG distribution. This is further supported by the observation that CA4+ does not accumulate in the chondrons in the most superficial parts of the cartilage. Zonal differences in GAG synthesis in chondrocytes has been reported, and this again might explain why CA4+ does not accumulate in the surface zone chondrocytes, which have lower GAG synthesis compared to the deeper zone (Archer, McDowell, Bayliss, Stephens, & Bentley, 1990; Aydelotte, Greenhill, & Kuettner, 1988; Siczkowski & Watt, 1990).

The results from the third sub-study also indicated that the time constants of the diffusion differ between the segmented chondron volume and ITM volume with all CA4+ concentrations. The unsegmented and ITM volume had similar time constants and the relative difference of the CA4+ intensities from the OA samples was less than 4%, indicating that low-resolution µCT studies are not greatly affected by CA4+ accumulation in chondrons.

An increase in GAG synthesis in the early stage of OA when compared to the normal and late stage has been reported (Lafeber, van Roy, Wilbrink, Huber-Bruning, & Bijlsma, 1992; Venkatesan et al., 2012), which could indicate that the CA4+ staining intensity would be increased in early OA patients. However, we did not find any correlation with the image intensities at any volume when compared to OA severity, evaluated from adjacent histological samples.

8.3 Analysis of osteocyte cellularity from µCT data (IV)

In the fourth sub-study, osteocyte cellularity was found to have a statistically significant correlation with individual and combinations of histogram and GLCM parameters determined from µCT. From the individual parameter correlations, GLCM had the strongest correlation with osteocyte cellularity, suggesting that the
local grey-level value association (GLCM correlation parameter) decreases in the µCT imaged subchondral bone when the cellularity increases. This observation was supported by the increase of GLCM contrast and decrease of GLCM IDM with increasing osteocyte cellularity, which indicates that local grey-level variation increases as local grey-level homogeneity decreases in the µCT imaged subchondral bone (see Table 4). This association was further increased when GLCM asymmetry information (GLCM cluster shade) was combined with the GLCM correlation parameter in the statistical model.

The utilisation of the texture and histogram-based analysis from µCT might bring further input to the existing µCT-based 3D histopathological grading system for osteochondral tissues (Nieminen et al., 2017). As this grading system is still subjective, µCT image analysis provides a possibility to create a more quantitative (and automated) osteoarthritis grading system.

It should be noted that the individual parameter correlations and combined models were not robust to the core extraction location. This brings an unfortunate limitation, as the osteocyte morphology is dependent on the surrounding bone matrix structure, which alters depending on the areal location in the tibial plateau. Consequently, the associations between osteocyte cellularity and the GLCM/histogram parameters from µCT images might be suppressed if the core extraction is not controlled.

8.4 Analysis of trabecular bone structure from CBCT data (IV)

The results of the fourth sub-study demonstrate a strong association of several histogram/GLCM parameters calculated from the CBCT data with the trabecular bone morphometrics. These results show promise in combining different GLCM/histogram parameters for obtaining more accurate estimates for trabecular morphometrics at micro-level. However, as the trabecular bone morphometrics, histogram parameters and GLCM parameters are co-dependent in their respective parameter groups, the interpretation of the results is challenging. Thus, our stepwise linear regression model and the further discussion is limited to the most relevant trabecular morphometric, i.e. BV/TV.

BV/TV had the strongest correlation with the histogram mean of all the individual GLCM/histogram parameter correlations. This was expected, as previous literature has shown a prominent association between the BV/TV of trabecular bone and the grey-level averages calculated from the ex vivo clinical resolution CT imaging of cancellous bone (Hsu et al., 2014). Furthermore, the
previous literature on the GLCM analysis of CT imaged bone (Diederichs et al., 2009; Mookiah et al., 2018; Nagarajan et al., 2015) encouraged the creation of a combinatory statistical model with the GLCM and histogram parameters to estimate BV/TV. In the current study, this resulted in even stronger correlation with BV/TV ($r = 0.932$) when compared to the highest correlation of the individual parameters.

The location-dependency of the individual histogram and GLCM parameters can be problematic, as the trabecular bone structure varies between the medial and lateral compartments (Roberts et al., 2017) as well as depth-wise (Johnston, Masri, & Wilson, 2009; Johnston, Kontulainen, Masri, & Wilson, 2010). However, the combination of the second-order statistics of GLCM parameters and first-order statistics of the histogram parameters resulted in a statistical model which was robust to the core extraction location.

It is important to note that the GLCM/histogram-based analysis might have relevance in the evaluation of osteoarthritis severity. Many of the GLCM/histogram parameters correlated with the OARSI grade. This is obviously a secondary association, as the OARSI grading is mainly based on the degenerative changes in articular cartilage tissue and the GLCM/histogram parameters describe only subchondral bone morphometrics. However, similarly as in the previous study (Finnilä et al., 2017), a significant association between trabecular bone morphometrics defined with µCT and OARSI grading was observed in the current study. Therefore, in principle the GLCM/histogram parameters could be utilised in the classification of OA severity from the clinical CT bone data.

8.5 Limitations

There were several limitations of this thesis work. In the first sub-study, the sample size was small ($n = 12$ from three animals, and $n = 4$ from two TKA patients) and the core diameters were different between the equine and human samples. Furthermore, the results from the correlation analysis of sufficient PTA staining time could be prone to collinearity.

In the second sub-study, the biological variation was relatively high, generating an 8% up to 26% difference in measured values through the different modalities.

In the third sub-study, several limitations were primarily related to the sample selection. The wide age range and lack of healthy subjects might limit the statistics in this relatively small sample size ($N=14$). Moreover, the grading system might not represent the best possible reference in this study, as the OARSI grading system
evaluates the progression of OA based mainly on cartilage matrix degradation, giving only a low emphasis to GAG depletion or increased synthesis of the GAGs.

In the fourth sub-study (IV), since only cores from four TKA patients/cadavers were studied (excluding femoral samples), the sample size in the areal location subgroup was relatively small and the sub-grouping of cadavers vs. TKA patients was skewed. Furthermore, the fourth sub-study was an *ex vivo* study, thus applying the GLCM/histogram analyses *in vivo* would include noise from the increased scattering of X-rays due to the surrounding soft tissues and increased volume of the object. However, the combination of the first- and second-order grey-level statistics could be advantageous in reducing the effect of noise in *in vivo* clinical CT analysis.

## 8.6 Micro-computed tomography – quantification tool for OA features from osteochondral tissues

This thesis presents an encompassing range of novel µCT methods for quantifying compositional and structural alterations of articular cartilage and subchondral bone. Alterations in the collagen matrix, GAG content and chondrocytes are the key features for histological grading systems for evaluating the severity of OA (Mankin et al., 1971; Pritzker et al., 2006). Moreover, alterations to subchondral bone structure are linked to OA (Burr & Gallant, 2012; Chou et al., 2013; Finnilä et al., 2017; Henrotin et al., 2012; Jaiprakash et al., 2012; Yan et al., 2018). This thesis introduced and/or validated CEµCT methods for quantifying all these key features in human articular cartilage (I-III). Furthermore, a method for evaluating sub-micro-structures (osteocyte cellularity) from µCT data was introduced (IV) as an addition to the existing methods measuring the microstructural morphometrics from µCT data (Bouxsein et al., 2010).

GAG depletion and chondrocyte proliferation are one of the first cartilage alterations in OA (Buckwalter & Martin, 2006; Mankin et al., 1971; Pritzker et al., 2006). Many of the current CEµCT methods are focused on GAG quantification using anionic contrast agents (Mittelstaedt & Xia, 2015; Palmer et al., 2006; Silvast et al., 2009). However, cationic contrast agents, including CA4+, have not only been shown to be more accurate for GAG content quantification (Bansal et al., 2011), but this result of this thesis shows that CA4+ has potential for investigating chondrons. Thus CA4+ is not only a promising tool for quantifying GAG content in human articular cartilage, but it could also be utilised to study chondron distribution in articular cartilage. The mechanisms of CA4+ accumulation in chondrons remains to be elucidated, but investigation of the micro-distribution of
CA4+ with high-resolution µCT could also be used in identifying chondrons with increased GAG synthesis from OA cartilage.

Fibrillation, clefts and disruption of the collagen matrix in the deeper zones of articular cartilage could be quantified with the novel CEµCT method using PTA. The aforementioned collagen matrix degradation features are usually an indication of early or middle stage OA in the histological grading systems, based on the progressive degeneration of cartilage (Mankin et al., 1971; Pritzker et al., 2006). Thus, CEµCT with PTA provides a comprehensive quantification tool for the structural alterations in articular cartilage caused by OA. Prior to sub-study I, collagen matrix quantification with CEµCT had been conducted by investigating the diffusion of the anionic Hexabrix® (Kokkonen, Jurvelin, Tiitu, & Töyräs, 2011; Kulmala et al., 2012). In these studies, the mechanical injury or collagen orientation could be estimated, but the measurements were indirect as they were based on the diffusion of a contrast agent the distribution of which varies with the GAG content. PTA, on the other hand, is suitable for the direct quantification of the topography of the articular cartilage surface, as well as deeper-zone collagen matrix disruptions, within human articular cartilage.

Microstructural alterations in the subchondral bone during OA have been extensively studied using native µCT imaging (Chou et al., 2013; Finnilä et al., 2017; Yan et al., 2018). In contrast to histopathological grading, which indicates that the subchondral bone is altered after the cartilage erosion (Pritzker et al., 2006), these studies suggest that the structural alterations are occurring already during the progressive degradation in cartilage caused by OA. Thus, it seems that the subchondral bone morphometrics can provide quantitative information about OA at any stage of the disease. The GLCM and histogram-based analysis methods investigated in this thesis broaden the subchondral morphometrics beyond the microstructure. As the quantification of osteocyte cellularity often requires SRCT, the presented analysis methods bring sub-microstructural morphometrics available to the cheaper and widely available µCT systems. Furthermore, these methods seem to be translatable to clinical CT systems.

The methods presented in this thesis provide a new set of tools for quantifying the relevant structural and compositional alterations of the articular cartilage and subchondral bone in vitro. Furthermore, these methods provide quantifiable metrics at all stages of OA. They provide a strong base for the further development of µCT-based 3D histopathology.
8.7 Future prospects

The first and second sub-studies demonstrated that PTA is a tool capable of staining the collagen content of articular cartilage in a CEµCT setup. As proposed in the sub-studies, a 3D histopathological grading system of OA has been developed based on PTA-based CEµCT (Nieminen et al., 2017). As that grading system is based on subjective evaluation, more quantitative approaches should be studied to evaluate OA severity with µCT. Combining staining protocols with CA4+ (which staining protocol for human articular cartilage was presented in III), and with GLCM/histogram analysis of subchondral bone, could result in comprehensive and quantitative 3D histopathological evaluation of OA severity. The methods presented in this thesis already provide quantitation of several osteochondral tissue features which are known to alter in the progress of OA, e.g. collagen content (PTA), GAG content and chondron distribution (CA4+), and osteocyte cellularity (GLCM/histogram-based analysis). Applying statistical methods of automated decision-making (“machine learning”) to this multiparametric data could generate automated and more accurate prediction or classification models for 3D histopathological grading.

Similarly, the clinical relevance of the presented GLCM/histogram-based analysis of CBCT-imaged bone could be reached with “machine learning”. However, further studies of the applicability of GLCM/histogram-based analysis to the in vivo CT imaging setup and larger data collection are still required.
9 Summary and conclusions

This thesis presents novel μCT methods to quantify osteochondral micro-structures related to osteoarthritis modifications to articular cartilage and subchondral bone. Novel approaches in the quantification of collagen content and the micro-distribution of GAG content were presented here for CEμCT studies of human articular cartilage. Furthermore, analysis methods for sub-resolution bone features from μCT and clinical resolution CBCT were introduced. The main conclusions of the present thesis are summarised below:

1. PTA distribution in CEμCT imaging is specific to the collagen content distribution in articular cartilage.
2. CA4+ accumulates in chondrons and its micro-distribution in human articular cartilage is related to the GAG content.
3. Osteocyte cellularity is associated with the texture and histogram parameters calculated from μCT imaged subchondral bone.
4. Sub-resolution trabecular bone features are strongly associated with the texture and histogram parameters calculated from CBCT-imaged subchondral bone.
References


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Original publications


Reprinted with permission from Elsevier (I, IV), PLOS (II), and Frontiers (III).

Original publications are not included in the electronic version of the dissertation.


1481. Kelloniemi, Annina (2018) Novel factors regulating cardiac remodeling in experimental models of cardiac hypertrophy and failure


1490. Tervasmäki, Anna (2018) Hereditary predisposition to breast cancer—evaluating the role of the rare copy number variant, protein-truncating and missense candidate alleles


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Sakari Karhula

QUANTIFICATION OF OSTEOCHONDRAL TISSUE MODIFICATIONS DURING OSTEOARTHRITIS USING MICRO-COMPUTED TOMOGRAPHY