Chondrocytes respond to mechanical stimuli by regulating their synthesis, but fail to do so during osteoarthritis, leading to articular cartilage degeneration. In this thesis, we used a partial meniscectomy rabbit model of knee osteoarthritis to study how cell biomechanics and articular cartilage structure are altered as early as 3 days after surgery. The findings provide valuable information, which can be useful for understanding, predicting and preventing osteoarthritis progression.
Ari Ronkainen

BIOMECHANICAL RESPONSES OF CHONDROCYTES IN HEALTHY AND MENISCECTOMIZED RABBIT KNEE JOINTS

ACADEMIC DISSERTATION

To be presented by the permission of the Faculty of Science and Forestry for public examination in the Auditorium SN200 in Snellmania Building of the University of Eastern Finland, Kuopio, on June 8th, 2018, at 12 o’clock.

University of Eastern Finland
Department of Applied Physics
Kuopio 2018
ABSTRACT

Articular cartilage is a hydrated soft tissue found at the bony ends of the diarthrodial joints. Under normal conditions, articular cartilage displays an exceptional load-carrying capacity allowing near frictionless contact between the articulating bones. The two main macromolecules found in the dense extracellular matrix of the articular cartilage are proteoglycans and collagen. Chondrocytes are the only cell type in the articular cartilage; these cells sparsely reside in this collagen-proteoglycan mesh and they are solely responsible for remodeling of the internal cartilage throughout the lifespan. Because chondrocytes are able to sense mechanical cues in their surroundings, changes to joint loading evoke alterations in the properties of the cartilage. However, it is poorly understood the extent to which the biomechanical responses of chondrocytes in distinct knee joint regions differ in their responses to cartilage loading, and how local cartilage structure, composition and mechanical properties are linked to this phenomenon. Even less is known how, and if, chondrocyte deformations become altered very soon after damage to the knee joint. This is important information, as alterations to the mechanical cues that chondrocytes experience in their microenvironment will likely disturb cartilage homeostasis.

In the present study, cell-tissue interrelationships were studied by measuring cartilage composition and chondrocyte deformations in response to mechanical loading in different rabbit knee joint locations, including patella, femoral groove, lateral and medial femoral condyles and lateral and medial tibial plateaus. The tested knee joint tissues were harvested from healthy rabbits and from rabbits that had undergone partial resection of the anterior horn of the lateral meniscus 3 days before sacrifice. After harvesting the tissues, a custom-built confocal laser scanning microscope equipped with an indentation system was used to capture in situ deformation response of superficial chondrocytes to a standardized indentation loading. After the confocal microscopic evaluation, the tissues were processed for histology to quantify the cartilage's collagen content, proteoglycan content and collagen orientation angle at the site of indentation, by applying microscopic and spectroscopic methods. Finally, axisymmetric multi-scale finite element models with measured cartilage properties were generated. An experimental indentation loading protocol was simulated in the models and cell volume changes due to indentation were compared to experimental values. Particular emphasis was placed on how chondrocyte deformations vary between knee joint sites, how cartilage composition is linked to the cell deformations and what are the early effects of surgery on the cell deformations, cartilage composition, and their interrelationship.

The superficial chondrocyte deformations evoked by similar loading conditions varied between knee joint sites and followed the local tissue strains. The
ABSTRACT

Articular cartilage is a hydrated soft tissue found at the bony ends of the diarthrodial joints. Under normal conditions, articular cartilage displays an exceptional load-carrying capacity allowing near frictionless contact between the articulating bones. The two main macromolecules found in the dense extracellular matrix of the articular cartilage are proteoglycans and collagen. Chondrocytes are the only cell type in the articular cartilage; these cells sparsely reside in this collagen-proteoglycan mesh and they are solely responsible for remodeling of the internal cartilage throughout the lifespan. Because chondrocytes are able to sense mechanical cues in their surroundings, changes to joint loading evoke alterations in the properties of the cartilage. However, it is poorly understood the extent to which the biomechanical responses of chondrocytes in distinct knee joint regions differ in their responses to cartilage loading, and how local cartilage structure, composition and mechanical properties are linked to this phenomenon. Even less is known how, and if, chondrocyte deformations become altered very soon after damage to the knee joint. This is important information, as alterations to the mechanical cues that chondrocytes experience in their microenvironment will likely disturb cartilage homeostasis.

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The superficial chondrocyte deformations evoked by similar loading conditions varied between knee joint sites and followed the local tissue strains. The
site-specific tissue responses were, as expected, related to differences in the composition and structure of the cartilage between the various knee joint sites. The orientation of the collagen fibril network and the amount of collagen in the local matrix around the chondrocytes were the two structural parameters most strongly related to chondrocyte deformations. Chondrocyte deformation behavior was altered and a loss of superficial proteoglycan content was observed 3 days after partial meniscectomy in all locations, except the lateral tibial cartilage. However, Young’s moduli and strains in cartilage, and the properties of the collagen network, were not significantly different between the surgery and control groups. In addition, the proteoglycan loss in the extracellular matrix could not solely explain the altered chondrocyte deformations. Finite element models were able to reproduce the experimental cell deformations with rather good accuracy, and predicted similar trends in the group-differences as those which were observed experimentally. The cell volume changes in the models displayed high sensitivity to local collagen orientation angle, consistently with the experimental analyses. Alterations to the proteoglycan content in either the pericellular matrix surrounding the cell or in the cartilage extracellular matrix had less extensive effects on cell volume changes, also in agreement with previous studies and experiments.

In conclusion, it is evident that the properties, structure and composition of cartilage are different between various knee joint locations, most likely reflecting the tissue’s adaptive capabilities to tolerate normal physiological loading. The naturally developing structured collagen network seems to be very important for determining the cellular biomechanics; this property can be crucially important when designing tissue engineered replacements for cartilage defects. However, alterations in chondrocyte biomechanics and the cartilage proteoglycan content seems to precede other micro-level structural changes. This means that in rabbits, the early proteoglycan loss potentially predisposes the collagen network to damage as early as 3 days following a partial meniscectomy. Hence, it could be possible to slow down the cartilage degeneration after surgery if normal levels of proteoglycan could be maintained in the tissue.

National Library of Medicine Classification: QT 34.5, WE 300, WE 348, WE 870

Medical Subject Headings: Cartilage, Articular; Cell Size; Chondrocytes; Collagen; Densitometry; Extracellular Matrix; Femur; Finite Element Analysis; Knee Joint; Meniscectomy; Microscopy, Confocal; Models, Animal; Osteoarthritis, Knee; Patella; Proteoglycans; Rabbits; Spectrum Analysis; Stress, Mechanical; Tibia

Yleinen suomalainen asiasanasto: biomekaniikka; elementtimenetelmät; fluoresenssimikroskoopia; kani; koe-eläinmallit; kollageeniit; kuormitus; nivelrikko; nivelruusto; numerinen analyysi; polven nivelkierukat; spektroskoopia; solu; soluvillaine; valomikroskoopia
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Ari Ronkainen
Kuopio, April 18, 2018
LIST OF PUBLICATIONS

This thesis consists of the present review of the author’s work in the field of chondrocyte and cartilage biomechanics and the following selection of the author’s publications:


Throughout the overview, these papers will be referred to by Roman numerals.

AUTHOR’S CONTRIBUTION

The publications selected in this dissertation are original research papers on the effect of partial meniscectomy on rabbit knee joint cartilage composition and structure, and on chondrocyte deformations due to mechanical loading. The author was the main contributor to studies II and IV and made a significant contribution to studies I and III. The author performed all microscopic and spectroscopic measurements and statistical analyses regarding cartilage extracellular matrix in studies I–IV. The author did not perform confocal microscopy or digital densitometry at high magnification, but made a significant contribution to the cell deformation data analysis and performed the digital densitometry analysis. The author performed most of the finite element analyses in study IV, whereas the co-authors had a larger role in the initial creation and development of the model. The author was the main writer in studies II and IV and a co-writer in studies I and III. Contributions from co-authors have been significant in each study.
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## ABBREVIATIONS

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACLT</td>
<td>Anterior cruciate ligament transection</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
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<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
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<tr>
<td>CZ</td>
<td>Calcified zone</td>
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<tr>
<td>DD</td>
<td>Digital densitometry</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>DMOAD</td>
<td>Disease modifying osteoarthritis drug</td>
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<tr>
<td>DPE</td>
<td>Dual-photon excitation</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellularly activated kinase</td>
</tr>
<tr>
<td>FACIT</td>
<td>Fibril-associated collagen with interrupted triple helix</td>
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<tr>
<td>FCD</td>
<td>Fixed charge density</td>
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<tr>
<td>FE</td>
<td>Finite element</td>
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<tr>
<td>FEA</td>
<td>Finite element analysis</td>
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<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor-2</td>
</tr>
<tr>
<td>FRPES</td>
<td>Fibril-reinforced poroelastic swelling</td>
</tr>
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<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>FTIRI</td>
<td>Fourier transform infrared imaging</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
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<tr>
<td>KS</td>
<td>Keratan sulfate</td>
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<tr>
<td>LMM</td>
<td>Linear mixed model</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MPM</td>
<td>Multi-photon microscopy</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MZ</td>
<td>Middle zone</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PCM</td>
<td>Pericellular matrix</td>
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<tr>
<td>PG</td>
<td>Proteoglycan</td>
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<tr>
<td>PLM</td>
<td>Polarized light microscopy</td>
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<tr>
<td>PM</td>
<td>Partial meniscectomy</td>
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<tr>
<td>REML</td>
<td>Restricted maximum likelihood</td>
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<tr>
<td>RVD</td>
<td>Regulatory volume decrease</td>
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<td>SLRP</td>
<td>Small leucine-rich repeat proteoglycan</td>
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<td>SZ</td>
<td>Superficial zone</td>
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<tr>
<td>TKR</td>
<td>Total knee replacement</td>
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<tr>
<td>VTK</td>
<td>Visualization toolkit</td>
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**SYMBOLS**

- $a$: Indenter radius
- $a_0$: Material constant
- $B$: Unstandardized regression coefficient
- $C$: Density ratio between primary and secondary fibrils
- $C$: Stiffness tensor
- $C$: right Cauchy-Green deformation tensor
- $c^-$: Concentration of mobile anions
- $c_{\text{ext}}$: External salt concentration
- $c_F$: Fixed charge density
- $c_{F,0}$: Initial fixed charge density
- $d$: Distance between cells after loading
- $d_0$: Distance between cells in unloaded state
- $d_{\text{vec}}$: Superficial zone thickness
- $e$: Void ratio
- $e_0$: Initial void ratio
- $\vec{e}_f$: Fibril orientation vector
- $\vec{e}_{f,0}$: Initial fibril orientation vector
- $E$: Young's modulus
- $E_f$: Fibril modulus
- $E_{nf}$: Non-fibrillar matrix modulus
- $E_{\text{measured}}$: Uncorrected Young's modulus (indentation)
- $F$: Deformation gradient tensor
- $G$: Bulk modulus
- $h$: Sample thickness
- $I$: Intensity of emerging light
- $I_{0^\circ - 90^\circ}$: Intensity of light with polarizer pair at $0^\circ - 90^\circ$
- $I$: Unit tensor
- $J$: Jacobian determinant
- $k$: Hydraulic permeability tensor
- $k$: Scalar hydraulic permeability
- $k_0$: Initial permeability
- $K$: Shear modulus
- $M$: Permeability strain-dependency coefficient
- $n_f$: Fluid volume fraction
- $n_{f,0}$: Initial fluid volume fraction
- $n_s$: Solid volume fraction
- $N_{f,\text{tot}}$: Total number of fibrils
- $p$: Fluid pressure
- $r$: Radius or correlation coefficient
- $r_{\text{vec}}$: Bending radius of middle zone fibrils
- $R$: Molar gas constant
- $S_{0-4}$: Stokes parameters
- $T$: Absolute temperature
- $T_c$: Chemical expansion stress
- $V$: Cell volume after loading
- $V_0$: Cell volume in unloaded state
- $W$: Strain energy density function
α_{PCM} Scaling factor for pericellular fixed charge density
β Standardized regression coefficient
γ_{ext} External activity coefficient
γ_{int} Internal activity coefficient
Δπ Donnan swelling pressure gradient
ε Strain tensor
ε_f Fibril strain
θ Polarization ellipse angle
θ_{col} Collagen orientation angle
κ Material constant or scaling factor
μ_f Water chemical potential
ν Poisson's ratio
ν_{nf} Poisson's ratio of the non-fibrillar matrix
ρ_z Depth-dependent fibril volume fraction
σ_{f,p} Primary fibril stress
σ_{f,s} Secondary fibril stress
σ_{eff} Effective solid stress tensor
σ_f Stress tensor of the fibrillar matrix
σ_{f,i} Stress tensor in i'th fibril
σ_f Stress tensor of the fluid
σ_{nf} Stress tensor of the non-fibrillar matrix
σ_s Stress tensor of the solid matrix
σ_{tot} Total stress tensor
ϕ_{ext} External osmotic coefficient
ϕ_{int} Internal osmotic coefficient
∇ Gradient
T Transpose
\| \| Norm
Introduction

There are three types of cartilaginous tissues in the human body; hyaline-, elastic- and fibro-cartilage. This thesis focuses on the most common type of hyaline cartilage – articular cartilage. This highly specialized connective soft tissue covers the ends of bones in the synovial joints, such as in the knee or hip joints. The main function of articular cartilage is to distribute loads so that they can be transmitted throughout the joint, while allowing near-frictionless gliding of the articulating surfaces past each other, even under extreme pressures of multiple body weights. These functions are attributable to the highly ordered molecular structure and composition of articular cartilage's extracellular matrix (ECM) together with the synovial fluid that is secreted into the joint cavity by the synovial membrane. The ECM of articular cartilage can be thought of as a biphasic material comprising both solid and fluid phases that act together as a whole system. The solid phase is provided with a structure by the highly oriented collagen fibril network that enmeshes negatively charged proteoglycans and includes the single type of cell found in articular cartilage, i.e., chondrocytes. The fluid phase is mainly water with dissolved ions. The negatively charged ions between proteoglycans and the ions within the fluid give rise to repulsive forces which can also be considered as a third phase, leading to a triphasic material model of cartilage. The chondrocytes are responsible for tissue remodeling, and hence, these cells are important regulators of articular cartilage health. [1–5]

During the most common musculoskeletal disease, osteoarthritis (OA), cartilage slowly degenerates and ultimately wears out completely [6]. The first macroscopic signs of OA appear in the articulating surfaces; these include collagen fibrillation, proteoglycan loss and an increase in the cartilage's water content [7, 8]. Concurrently, chondrocytes show signs of increased proliferation, cluster formation and overall accelerated synthesis of both matrix proteins and matrix-degrading enzymes [9]. However, during OA, the rate of catabolism exceeds that of anabolism and degradation of ECM constituents follows [10]. The changes that occur during OA in the joint include bone remodeling, osteophyte formation and synovial inflammation [4, 6, 9, 10]. It is these changes that cause joint stiffness, pain and loss of function, which can make daily life activities challenging or even lead to early retirement from employment [11]. The disease is multifactorial with age, obesity and joint trauma as the main risk factors and thus the prevalence of OA has steadily risen over the past years with no apparent sign or indication that this trend will reverse. However, not all elderly people develop OA during their lifetime, indicating that OA is not an inherent part of aging [6, 11, 12]. The challenge in treating OA is that early disease is virtually asymptomatic; symptoms only become manifested when substantial, and usually irreversible, structural damage has already taken place. As we still lack structure-modifying drugs for OA and pain medication can only relieve but not reverse the symptoms, it is not unusual that end-stage OA demands joint replacement surgery [12].

Moderate mechanical loading is required to ensure cartilage homeostasis, whereas both overuse and disuse (e.g., immobilization) may lead to cartilage degeneration.
1 Introduction

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Moderate mechanical loading is required to ensure cartilage homeostasis, whereas both overuse and disuse (e.g., immobilization) may lead to cartilage
degradation [6, 13]. For these reasons, animal model studies utilizing destabilization surgeries such as meniscectomy or cruciate ligament transection are commonly exploited to initiate progression of post-traumatic OA in a controllable manner [14–17]. It has been observed that cartilage cells i.e., chondrocytes, actively sense and respond to loads by altering the molecules that they synthesize, as well as changing their differentiation, phenotype and rate of senescence [18–20]. These changes take place through multiple biological pathways and are regulated not only by ECM composition but also by the pericellular matrix (PCM) surrounding the chondrocytes, which has a distinguishably different composition and function as compared to the bulk ECM [21, 22]. It has also been reported that not only the loading amplitude, but also frequency, duration and velocity affect the resulting cell response [23]. Since deformation of chondrocytes is one of the pathways involved in sensing mechanical cues, the deformation behavior of chondrocytes has been measured with various in vivo, in situ and in vitro settings, with computational models also being utilized for the same purposes [24–29]. The use of computational methods makes possible the investigation of variables that are experimentally impossible, or extremely challenging, to measure [29], such as stresses, strains and fluid flow around the chondrocytes, and their contribution to chondrocyte deformation. It is also possible to monitor the extent to which these variables display temporal or spatial variations in the situation that cartilage is damaged and its material properties have become altered, for example, due to OA.

Depth-dependent properties within articular cartilage, such as those within the collagen network and the fixed charge density associated with the proteoglycans are important for cellular mechanics [30]. Changes in these depth-dependent properties are the hallmarks of cartilage degeneration [31–33]. However, it is currently poorly understood the extent to which cellular deformations are altered early after tissue damage. In addition, it has not been comprehensively examined how much variation there is in the deformation behavior of chondrocytes within different anatomical locations in the knee joint, and the extent to which the local ECM and PCM composition affect the chondrocyte deformations. A better understanding of the aforementioned properties could help in identifying the molecules or structures in cartilage that could be targeted in order to maintain healthy homeostasis in the tissue.

Hence, in this thesis, chondrocyte deformations due to static indentation loading were studied in situ using customized state-of-the-art confocal microscopy that allowed simultaneous mechanical loading and imaging of cartilage. The experiments were performed in multiple rabbit knee joint locations, both in healthy tissue and in the same tissue at 3 days after a partial lateral meniscectomy. One particular focus was to clarify how local structure and composition of cartilage affects the chondrocyte deformations; and to examine if this is already altered at 3 days after the surgery. In addition to the experimental measurements and analyses, finite element modeling was performed to make a theoretical contribution to understanding how the different cartilage constituents impact on cellular deformation.
2 Knee joint articular cartilage

Diarthrodial joints, such as the knee joint, are enclosed by a fibrous capsule, which has its inner surface lined with a metabolically active synovium (Figure 2.1). The synovium secretes synovial fluid into the joint space and absorbs metabolic waste products [34]. Other structures in the knee joint include articular cartilage, lateral and medial menisci, and anterior and posterior cruciate ligaments (Figure 2.1). The movement of the knee joint is also stabilized and restricted by additional ligaments, tendons, muscles, fat pads and bursae (fluid filled sacs) [35,36]. The main interest of this investigation, articular cartilage, covers the bony ends of femur, tibia and patella (Figure 2.1). This smooth, glassy substance allows near frictionless articulation (μ ≈ 0.001) of the bones at the pressures evoked by loads which can be as large as several times an individual’s body weight [37]. Furthermore, under normal circumstances, articular cartilage can withstand, without failing, the undeniably hostile biomechanical environment of the knee joint for an individual’s lifetime. This is achieved by the exceptional structure and composition of the cartilage extracellular matrix (ECM), which is synthesized and maintained by the cartilage cells, i.e., the chondrocytes [38]. However, one of the peculiarities of cartilage is that it is alymphatic, avascular and aneural, which is reflected in its slow metabolism and poor repair capability. This means also that damage to cartilage does not directly cause any pain, but biochemical factors associated with cartilage damage are perceived by sensory neurons in the surrounding tissues [6]. For these reasons, cartilage injuries may go unnoticed and frequently progress to an untreatable state [39,40].

![Figure 2.1: Simplified presentation of the knee joint anatomy and a zoomed view of the zonal structure of the articular cartilage.](image-url)
2.1 EXTRACELLULAR MATRIX, COMPOSITION AND FUNCTION

Articular cartilage ECM can be considered to be a biphasic material with a fluid phase and an organic solid phase [41]. The fluid phase consists of water and dissolved ions, whereas the solid phase is essentially a collagen network of predominately type II collagen that enmeshes proteoglycans (PGs), other less common proteins and the chondrocytes (Figure 2.3). PGs contain high amounts of negatively charged groups (SO$_3^-$ and COO$^-$); these account for the high swelling pressure in the articular cartilage [42]. The swelling is due to electrostatic repulsion between these negatively charged groups and due to Donnan osmotic pressure, which arises from the imbalanced ion concentrations between articular cartilage and synovial fluid [5]. If one assesses it by weight, articular cartilage is mostly water, and the water content peaks in the superficial cartilage (∼ 85%) with the tissue becoming less hydrated towards the deep zone (∼ 65%). The water makes cartilage viscoelastic and, hence, cartilage deformation under loading depends on both the strain rate and strain magnitude [43].

2.1.1 Collagen network

The main function of collagen network is to provide tensile stiffness and to resist the swelling pressure attributable to the proteoglycans, which makes the collagen network pre-strained, and indirectly capable of providing compressive stiffness [44]. Furthermore, the orientation of collagen fibrils varies throughout the depth of the cartilage (Figure 2.1) and, based on this, cartilage can be divided into the superficial zone (SZ), the middle zone (MZ) and the deep zone (DZ). In the SZ, the fibrils are aligned in parallel to the cartilage surface and preferentially have an in-plane orientation [45]. In the MZ, the orientation of the collagen fibrils starts to arch towards the underlying bone, resulting in a “random” orientation. In the DZ, the fibrils are aligned perpendicular to the joint surface, forming the characteristic arcade-like orientation [46]. This zonal variation in orientation is usually cited as the Benninghoff’s arcade concept [47]. The in-plane orientation of SZ fibrils can be observed by inserting an ink stained needle into the cartilage – this reveals collagen fibrils splitting in certain directions (Figure 2.2), forming so-called split-lines [48]. These split-lines are postulated to orient along the principal stresses that occur in motion, and cartilage is known to be more resilient in that direction [49–53]. In addition, collagen fibrils become stacked together and form fibers of different size (Figure 2.3). There is also heterogeneity in this property, as the average diameter of collagen fibers increases as a function of cartilage depth, being 25–50 nm in the SZ, 60–140 nm in the MZ and up to 200 nm in the DZ, where they become anchored into the calcified zone (CZ) [54,55].

![Illustration of the split-line pattern in the femoral condyle](image)
Collagen is the generic name for the proteins that form a triple helix of three polypeptide chains (Figure 2.3), where each polypeptide chain is a repeat of glycine-X-Y [58] where X and Y are other amino acids, most often proline or hydroxyproline [58]. Glycine is required as the third component in every strand as it is the smallest of the amino acids, enabling tight packing of the collagen triple helix [58–60]. At least 28 different collagen subtypes are known to occur in vertebrates, and at least 8 of these have been identified in cartilage [60,61]. Type II collagen is the backbone and principal component of the cartilage collagen network, representing 60% – 80% of the cartilage dry weight and accounting for 80% – 95% of all collagens in cartilage with the percentage increasing with age [58–60]. In addition, at least collagen types III, VI, IX, X, XI, XII and XIV are present in cartilage [60].

The most important distinction in the case of articular cartilage is between the fibril-forming collagens (such as type II and type XI) and FACITs (fibril-associated collagens with interrupted triple helices), which do not form fibril networks, but bind to the surface of other fibrils [58]. The type II collagen fibrils are formed by the staggering of type II collagen molecules in a periodic pattern that can be observed with electron microscopy [60]. This fibril structure is further stabilized by covalent cross-linking with (mostly) type IX and XI collagens [60]. Type IX collagen is the archetypical FACIT, found periodically along the surface of type II collagen [58,61,62]. Type XI collagen, on the other hand, is required for the formation of type II fibrils, as this type restricts the diameter of formed fibrils [60,63,64]. It has also been recently suggested that the collagen matrix undergoes minimal turnover...
after skeletal maturity in humans, making the cross-linked type II collagen network virtually a permanent structure [65].

If one considers the other minor collagens, then type III collagen accumulates with age and tissue damage, and is most prominent in the superficial cartilage. Its functions are not well understood, but it has been suggested to modify fibril network in response to damage to the matrix [66,67]. Type VI collagen is another of the widely studied collagen types, as it is exclusively concentrated in the pericellular matrix (PCM), where it anchors the cell to the surrounding matrix and probably participates in cell signaling. Its abundance reveals both spatial and temporal variation during OA [68–71]. Type X collagen is the most abundant collagen in calcified cartilage, where its expression precedes endochondral ossification. In addition, type X collagen has been proposed to stiffen the type II collagen network [58,59,72,73]. Both type XII and XIV collagens belong to the FACIT subfamily. Type XII is abundant in areas with highly ordered fibrils (such as in the superficial layer); this form is suggested to stabilize these structures [60,74]. On the other hand, type XIV is uniformly distributed in articular cartilage, although being more abundant in areas of high mechanical stress, where it is suggested to affect tissue integrity and mechanical properties [75].

2.1.2 Proteoglycans

Proteoglycans (PGs) are another important proteins found in articular cartilage, accounting for about 20–30% of cartilage dry weight [76]. PGs are formed by a core protein, which has multiple sulfated glycosaminoglycan (GAG) chains attached into it (Figure 2.3). On a weight basis, aggrecan is the most abundant PG in cartilage and it has two kinds of GAGs attached to it – chondroitin sulfate (CS) and keratan sulfate (KS) chains [77]. Both KS and CS chains have SO₄ and COOH groups that have negative charges when they become ionized [4]. In addition, aggrecan monomers form large PG aggregates in the cartilage ECM [78]. This occurs with the help of link proteins that attach multiple aggrecan monomers side-by-side to the hyaluronic acid (HA) filament, forming huge aggregates that become trapped in the intrafibrillar space [56]. When present in an aqueous environment, aggrecans tend to swell in order to expand the amount of separation from one strand to the next. In cartilage, this swelling is restricted by the collagen network, and an equilibrium state is reached when tensile forces in the collagen network are balanced with the swelling pressure induced by the PGs [79].

When cartilage is loaded, the extracellular fluid can flow within and out of the cartilage ECM, however, this happens quite slowly as cartilage has a relatively low permeability due to the vast amount of PGs trapped within the collagen network [79]. When unloaded, the cartilage will swell again due to the osmotic pressure generated by the PGs. Hence, the high PG concentration of cartilage is one reason for the characteristic viscoelastic creep behavior of cartilage and makes it possible to achieve a fast tissue recovery after loading [80]. The PG content is not uniform; instead, it is lower in the SZ and gradually increases with cartilage depth [76]. It has been postulated that if the PG content was uniform, this would generate a very high osmotic pressure gradient between the SZ and synovial fluid, leading to a large tension in the superficial collagen network [80]. It is worth noting that in the most superficial cartilage, where the PG content is low and permeability within ECM is higher, the tightly packed collagen fibrils also efficiently restrict fluid flow out from the tissue [81].
In addition to the aggrecan aggregates, cartilage contains minor amounts of other proteoglycans important for tissue function. One family of these kinds of PGs is the SLRPs (small leucine-rich repeat proteoglycans) e.g., biglycan, fibromodulin, decorin and lumican [56]. The function of SLRPs depends on their structure and the attached GAG chains. Decorin, fibromodulin and lumican are known to interact with collagen fibrils, regulating the fibril diameter and protecting the fibrils from collagenases by occupying cleavage sites on the collagen molecule [56,77]. Biglycan, on the other hand, accumulates on the cell surface and pericellular areas, pointing to a role in cell signaling and differentiation [82]. There are also additional larger PGs present in cartilage, such as perlecan, which is known to interact with the cell surface and growth factors [83]. The mechanical role of these minor PGs is less well known, but knock-out mice studies have demonstrated that a lack of these minor PGs impairs the development of functional cartilage [56,77].

2.1.3 Interstitial water

As stated, water is the most abundant component of articular cartilage and its content is highest in the SZ; the concentration gradually decreases towards the subchondral bone [84]. A portion of the water, along with the ions dissolved in the water, is known to reside in the intra-fibrillar space of the collagen, but most of the water is present in the pores of the collagen-PG network [38,85]. Due to the high negative fixed charge of PGs, the interstitial fluid contains a very high concentration of positive cations which are needed to maintain the electrochemical balance in the tissue. The interstitial water can flow out and within the cartilage, which allows solutes from the synovial fluid to reach the cartilage. However, fluid flow is relatively slow in cartilage due to its low pore-size, and hence, its low permeability ($k \approx 10^{-15} \text{ m}^4/\text{Ns}$). For this reason, small solutes (such as oxygen and glucose) are predominantly transported by diffusion, which is approximately 10-100 times faster [86].

In addition to providing a nutritional pathway, water is crucial for the mechanical resilience of articular cartilage. When cartilage is dynamically compressed, the interstitial fluid instantly becomes pressurized and can carry as much as 95% of the load [87]. In addition, even under static loads, the fluid phase carries the majority of the load for many minutes [88]. Because of this, in order to ensure the inherent properties of the proteoglycan-collagen matrix, one has to compress cartilage until the equilibrium of no fluid flow is reached [76]. Under high compressive loads the permeability becomes anisotropic, and is decreased in the direction perpendicular to compression [89,90]. Since PGs, and their GAGs, are the main regulators of permeability, it was suggested (and validated in a theoretical model) that this effect could arise from the reorientation of GAGs due to compression [89]. Functionally, the decrease of permeability in this direction will improve the local load support by not allowing the escape of fluid beneath the contact area [90].

2.2 PERICELLULAR MATRIX

Chondrocytes are separated from the articular cartilage ECM by a thin pericellular matrix (PCM); this has a different biochemical composition and biomechanical properties compared to the bulk ECM [21,83,91–93]. There is evidence that the PCM also affects both the chemical and mechanical signals perceived by the
chondrocytes [91]. The entity formed by the PCM and the enclosed chondrocyte is often referred to in the literature as the chondron [25, 92, 94–96].

2.2.1 Biochemical composition

In addition to type II and IX collagen, type VI collagen is the most prominent molecular marker of the PCM [97, 98]. The PCM is also enriched with hyaluronic acid, link protein, aggrecan monomers and is rich in PGs such as biglycan and perlecan [95, 99]. The collagen network in PCM is thinner than in the surrounding matrix (Figure 2.4), but densely and tangentially packed around the chondrocyte, forming a basket-like enclosure [100]. The water content of the PCM has been postulated to be similar or somewhat higher than in the surrounding ECM [91, 95, 101–103]. Mice lacking type VI collagen still form a PCM that can be isolated, but the stiffness of the PCM in the knockout mice was reduced to one third of that of the wild type controls [104]. In addition, the knockout mice experienced an accelerated development of osteoarthritic joint degeneration [104]. However, no difference was observed in the cartilage stiffness between the knockout and wild type mice, strongly suggesting that type VI collagen exclusively can influence the properties of the PCM [104].

Figure 2.4: Helium ion microscope images from deep zone cartilage. Pericellular “basket” that houses chondrocyte is shown (A), with higher magnification image from the interior wall of the PCM (B). Figure adapted from [100] with kind permission from John Wiley & Sons, Inc.

Although there are many PCM proteoglycans, one large heparan sulfate (HS) proteoglycan, perlecan, has been identified as an important regulator of cell adhesion, cartilage development and cell differentiation [105]. Even though perlecan is mainly packed in the PCM, it likely has a role in the ECM development, as mice with a mutation in the perlecan encoding gene have a severely defective cartilage collagen network [106]. Other evidence for an important role is that the amount of perlecan is increased and up-regulated near to sites of damage in late stage osteoarthritis, evidence that it is involved in an attempt to repair or stabilize the defected cartilage [107]. Interestingly, perlecan seems to relate inversely to the elastic modulus of the PCM, as removal of HS chains with heparinase increased the PCM moduli in the interior PCM regions that are rich in perlecan [108]. Perlecan is also main factor that binds fibroblast growth factor-2 (FGF-2) in the PCM, suggesting a role in mechanotransduction, as activation of extracellularly regulated
kinase (ERK) due to tissue loading is known to be dependent on the pericellular FGF-2 concentration [109,110].

2.2.2 Biomechanical properties

The mechanical properties of the PCM can be directly measured by extracting chondrons from the tissue or by preparing sections of the tissue. Indirect approximations of properties can be attained through theoretical modeling, which requires deformations in the PCM to be measured in intact or explant tissue [21, 25, 91, 111]. There are techniques that can directly measure the mechanical properties of the PCM i.e., micropipette aspiration, compression of isolated chondrons, and more recently, the exploitation of atomic force microscopy (AFM) on tissue sections [93,94,112–115]. In addition, cell-seeded scaffolds can be used, as chondrocytes readily form a PCM in culture [25,102,116,117]. Varying results for the elastic modulus have been reported (19 – 162 kPa) as species, tissue health and sample preparation techniques affect the mechanical properties of the PCM [25,93,94,116]. One common observation, however, with all these setups is that the PCM is 1 to 2 orders of magnitude stiffer than the chondrocyte and the ECM is 1 to 2 orders of magnitude stiffer than the PCM [91,93,94]. In addition, the PCM restricts fluid flux as it has lower permeability than the surrounding ECM [118,119]. The PCM also affects the rupture behavior of the chondrocytes, as it has been observed that 85 ± 1% deformation and a 3.1 ± 0.3 μN force was required to rupture chondrons (compression between two parallel surfaces), compared to 78 ± 1% deformation and 2.1 ± 0.2 μN force which would be required to rupture single chondrocyte [120].

In summary, the PCM is an important buffer zone that protects the chondrocyte, and firmly attaches it to the surrounding ECM [116]. Interestingly, the PCM displays uniform stiffness between the different cartilage zones, whereas the stiffness of the ECM is known to gradually increase with cartilage depth [22,93,112,114,121]. This results in a reduced stiffness mismatch between the PCM and the ECM in the SZ of cartilage compared to the MZ and DZ, which causes zone-dependent strain amplification and stress shielding of chondrocytes [22,122]. In addition, when probing tissue sections with AFM, the PCM stiffness shows directional dependence in the SZ and MZ, being higher when measured in parallel to the local split-line direction [114]. However, this split-line dependency is not as obvious as in the local ECM [114].

2.3 CHONDROCYTES

As stated, chondrocytes are the cells present in articular cartilage; these cells have adapted to survive in a biomechanically hostile environment with the low oxygen tension present in cartilage [123]. Chondrocytes are metabolically active and take part in the synthesis and organization of new ECM molecules, as well as in the degradation and recycling of damaged molecules [4,124]. The cells can be affected by both mechanical and chemical signals in their microenvironment, and the conversion of mechanical signals into synthetic activity is termed mechanotransduction [19]. In human cartilage, the chondrocytes are sparsely distributed and occupy approximately less than 5% of total cartilage volume [125]. Due to the sparse distribution of these cells, the amount of matrix associated per cell is large compared to cell volume, ranging from 50,000 μm³ to 150,000 μm³.
Chondrocytes are more abundant in the superficial layers and their shape depends on their depth within the cartilage (Figure 2.5) [4,125]. In the SZ, the cells are flat and ellipsoidal with their long axis oriented along the local collagen fibrils. In the MZ, the cells appear more rounded, and as in the SZ, they form single-cell chondrons [125]. In the DZ, the cells form column-like structures, where multiple cells occupy a single chondron [126]. The volume of chondrocytes increases with depth, for example in the case of pig cartilage, the volume of the SZ cells was \( 416 \pm 121 \, \mu m^3 \) and it increased to \( 1117 \pm 287 \, \mu m^3 \) in the DZ [126]. At the same time, the ratio of PCM to cell volume varied between 2 and 4 but did not show any significant depth-dependency [126].

**Figure 2.5:** Chondrocyte shape changes significantly with cartilage depth, and in the deeper layers the column-like chondrons usually occupy multiple cells.

### 2.4 OSTEOPATHRITIS

Unfortunately, articular cartilage injuries are frequent, poorly treatable and can be responsible for the total loss of normal joint function [127]. Osteoarthritis (OA) is the most common pathological joint disorder and approximately 10–12% of the adult population suffer from symptomatic OA [11]. In 2012, it was estimated that in global terms, 250 million people suffer from knee OA, and data from 1990 to 2010 shows that the years lived with disability due to knee and hip OA increased by 64% [128]. The estimates of the prevalence of OA make future estimates even more troublesome, as the number of people affected by OA will increase by about 50% over the next 20 years [11,129]. The increasing prevalence will be accompanied by a growing financial burden both for nations and individuals. For example, this can be seen from the increasing trend of aggregate hospital costs attributable to OA in the United States (Figure 2.6).

OA is characterized by the progressive loss of articular cartilage (Figure 2.7), but it also involves alterations in the surrounding structures, for example, ligament laxity, synovial inflammation, subchondral bone remodeling and osteophyte formation at the joint margins [7]. The loss of cartilage can also be accompanied by the formation of repair tissue, which is usually not identical to the properties to native tissue [130]. OA usually evolves slowly with the first macroscopic sign being...
the fibrillation of the normally smooth articular cartilage surface [7,130]. However, the loss of superficial PG content and an increase in the cartilage water content, accompanied by tissue softening, seems to precede any visually observable changes [40]. During OA, chondrocytes become active, seen as cell proliferation, cell cluster formation and increased cell synthesis [131]. Complex matrix remodeling takes place as various catabolic and anabolic factors are competing together [10, 40, 131]. This stage of the disease can last for years but usually ultimately it leads to irreversible destruction of the cartilage matrix [131, 132]. Alterations in articular cartilage are accompanied by a thickening of the subchondral bone, cyst development in the subchondral bone and new bone formation at the joint margins [40]. Other bony changes include alterations to the bone mineral content, increased subchondral bone vascularization, changes in the volume fraction of trabecular bone and the development of bone marrow lesions [131].

In patients, the common symptoms of OA are chronic and intermittent pain, activity limitations, poor sleep, fatigue and overall poor quality of life [11]. It is known that disease modifying OA drugs (DMOADs) could be one choice to treat early OA, when cartilage is still mostly intact but the joint is in a state of inflammation and catabolism [133]. The DMOADs can be roughly divided by their postulated effect on cartilage into either growth promoting or damage preventing agents [134]. Anti-degradation and anti-inflammatory drugs have shown promising results in slowing down OA progression in animal models by targeting biological pathways such as those involved in cell differentiation, expression and activity of catabolic matrix metalloproteinases (MMPs) or by altering the amount of pro-inflammatory cytokines [135–138]. In addition, it has been observed that the selective application of growth factors promotes the integrity of repair cartilage, which too often develops into fibrous tissue lacking any proper mechanical properties [139, 140]. However, the major challenge for identifying a single DMOAD that would work in all cases is that the factors leading to the OA development can be biomechanical, metabolic, genetic, post-traumatic or a combination of these pathways [141]. Hence, if they are to be efficacious, DMOADs
there may also be areas where the cartilage seems to be macroscopically intact or extensively damaged and reflects the situation in the end-stage disease although joint replacement surgeries, and in this scenario, the joint as a whole is already [147]. To some extent, the only consistent source of human tissue is from knee fact that human articular cartilage, or joint tissues in general, are rarely available initiation and progression of OA [14, 15, 17, 147–151]. This is largely due to the Animal models have been commonly utilized in experimental investigations of the 2.4.1 Animal models shown promising results in animal models [144–146]. Because of these problems with TKR, integrative cartilage repair with surrounding tissues, which can both lead to the need for recurrent surgeries [144]. The inherent weakness with TKR is the limited lifetime of the implant and its integration with surrounding tissues, which can both lead to the need for recurrent surgeries [144]. The ultimate intervention usually is total knee replacement (TKR). However, timing of TKR can be challenging as it has been observed that functional outcomes are better if patients are operated before there is a total loss of knee joint function [143]. The inherent weakness with TKR is the limited lifetime of the implant and its integration with surrounding tissues, which can both lead to the need for recurrent surgeries [144]. Because of these problems with TKR, integrative cartilage repair with tissue-engineered grafts or different biomaterial scaffolds that would provide seamless integration with the surrounding tissue are being developed, and have shown promising results in animal models [144–146].

2.4.1 Animal models

Animal models have been commonly utilized in experimental investigations of the initiation and progression of OA [14, 15, 17, 147–151]. This is largely due to the fact that human articular cartilage, or joint tissues in general, are rarely available [147]. To some extent, the only consistent source of human tissue is from knee joint replacement surgeries, and in this scenario, the joint as a whole is already extensively damaged and reflects the situation in the end-stage disease although there may also be areas where the cartilage seems to be macroscopically intact or

![Figure 2.7: Progression of osteoarthritis with concurrent structural changes in articular cartilage are illustrated.](image)
only slightly fibrillated. While it could be argued that articular cartilage from these areas might be suitable for early OA studies, it must be appreciated that the cartilage from these regions has likely been exposed to abnormal mechanical and biochemical signals for years. For this reason, the only practical way to study early changes and temporal evolution in OA in a controlled manner is to use animal models [14]. There are many ways to induce OA in animal models, including surgery, chemicals, enzymes, genetic modification – some animals even develop OA spontaneously [16]. Each different model of OA has its own benefits and disadvantages, which have to be considered when drawing conclusions and considering their relevance to human OA. The smallest animals, such as mouse, rat, guinea-pig and rabbit are low cost, easy to house and may allow genetic manipulation. Larger animals, such as dog, goat, sheep, and horse, have more relevant anatomy and knee joint biomechanics, and have large enough joints which allow routine diagnostic imaging or on which one can perform arthroscopic surgeries and interventions [15]. Interestingly, the composition of cartilage between species of varying size is rather homogeneous, as a study of 58 different species, ranging from a mouse weighing 25 g to a 4000 kg elephant, reported that the proteoglycan content and collagen content per gram of cartilage were essentially unrelated to the mass of the studied species [152]. In addition, the chondrocytes seemed to be of a similar shape and volume in different species [153–156].

2.4.2 Rabbit model

In terms of anatomy, the rabbit knee joint is very similar to the human knee joint, only the patella is proportionally smaller in the rabbit [157]. The main differences are found in the gait, as the rabbit knee joint is kept in high flexion most of the time. The volume fraction of cells is higher in rabbit cartilage compared to human; and in general, the cell volume fraction is inversely related to the mass of the species under examination [15,152]. In addition, human articular cartilage is many times thicker than the corresponding tissue in the rabbit [157]. It should be noted that the higher number of cells per volume in smaller animals is linked to a higher regenerative capacity, and because of this, small animals may be poorly suited for experiments that aim to investigate cartilage repair strategies [158]. In addition, the age of the animal needs to be considered, for example, rabbits can have open physes on growth plates of femur and tibia until they are 8 months old, and at a younger age, the cartilage possesses a greater potential to regenerate from damage [15].

Anterior cruciate ligament transection (ACLT) and full or partial meniscectomy (PM) are widely used methods to create mechanical instability in the rabbit knee joint; these procedures lead to the progressive development of OA [159–165]. In humans, arthroscopic partial meniscectomy has been occasionally used to treat meniscal tears, although the outcomes have been questioned [166]. In addition, ACL rupture is a common sport-related injury [167]. Hence, both animal models can be used (with certain obvious limitations) also to study what could occur due to either a partial meniscectomy or the absence of ACL in human cartilage. In rabbits, damage to the superficial collagen network was observed in the scanning electron microscope at 2 weeks after a meniscectomy. At 12 weeks, erosion of cartilage up to subchondral bone was evident in the weight-bearing regions of femoral condyle [165]. In another study, osteophyte formation on femur and tibia was seen as early as 2 weeks after a partial meniscectomy, and these, with other evident macroscopic damages to cartilage, gradually deteriorated towards the
12-week time point [159]. Similarly, Lindhorst et al. (2005) reported macroscopically visible signs of surface fibrillation and significant histological alterations in cartilage 2 weeks after meniscectomy [161]. However, possibly the earliest reported changes have been the loss of proteoglycan staining which could be observed 3 days after a partial meniscectomy, followed by superficial fibrillation, erosion and ulcer formation only 1 week after surgery in femoral and tibial cartilages [163]. In that study, temporal damage progression was also evident. In contrast to the other studies, Hoch et al. (1983) observed a loss of PGs with simultaneous decrease in equilibrium modulus, which both peaked in magnitude at 2 weeks after a medial meniscectomy, but the alterations were reversible and returned towards normal values during the 6 month follow-up [168]. However, this observation of spontaneous regeneration after meniscectomy should be viewed with caution, as some of the animals were rather young (6–9 month old animals) and only 1–2 animals were available at each time point after 6 weeks. It should be noted that post-surgical inflammation due to cutting the capsule and synovium is not likely alone sufficient to evoke the observed changes in these rabbit models, as previously various sham-groups have been reported to be unaffected [159,163,165,168].
3 Cell-tissue interactions

Mechanical forces caused by movement maintain cartilage, bone, muscle and tendon tissue remodeling throughout the lifespan [19]. Articular cartilage chondrocytes are exposed to variety of mechanical and chemical mediators, for example, growth factors, hormones, cytokines, stresses, strains, electrical currents and hydraulic pressure [1]. Significant alterations to any of these signals can potentially alter the balance of matrix anabolism and catabolism, which can lead to articular cartilage degeneration and osteoarthritis [10].

Mechanotransduction, i.e., the way in which the mechanical signals are converted to biochemical responses, is a complex phenomenon due to the multiphase nature of articular cartilage and its complex response to loading [169]. During mechanical loading, the interstitial fluid in articular cartilage tries to squeeze out from the solid collagen-proteoglycan matrix [1]. Due to the low permeability of cartilage, large frictional drag forces are exerted on the solid ECM and cells, and the fluid becomes pressurized. Concurrently, as proteoglycan aggregates are restrained in the ECM, they become closer to each other during cartilage compression and there is an increase in the repulsive charge-to-charge electrostatic forces between the negatively charged GAG chains [170]. In addition, dissolved ions in the interstitial fluid are moving within this charged ECM, causing streaming and diffusion potentials [5]. During prolonged loading, the fluid flow finally ceases and the load carried by the fluid pressurization becomes borne by the solid collagen-proteoglycan mesh. Chondrocytes are known to sense all of these mechanical cues caused by cartilage deformation together with the subsequent changes in ECM ionic composition, hydrostatic pressure and streaming potentials [4,123,124,171–173]. Therefore, it is not surprising that experiments with in vitro cartilage plugs have demonstrated that cellular responses are highly sensitive not only to the magnitude of the load, but also to the loading pattern, including frequency, velocity and waveform of the loading [174–176].

3.1 RESPONSE OF CHONDROCYTES TO LOADING

Similar to cartilage properties, also the deformation behavior of chondrocytes during mechanical loading depends on the cartilage zone [24,122,177]. Superficial chondrocytes show the highest degree of anisotropy, expanding more in parallel to the local cartilage split-lines [24]. In addition, the deformation behavior of chondrocytes is different in native ECM than in agarose gel cultures or in other in vitro conditions [24, 117, 178, 179]. Generally, chondrocytes lose volume and decrease in height under axial compression while simultaneously expanding laterally [24, 122, 155, 179]. The cell volume can, however, also increase after mechanical loading, as has been observed to occur in the rabbit model of OA [31,32,177]. It has also been observed that strain transfer from the ECM to the PCM, and further to the chondrocyte is non-linear; it occurs in such a way that small strains become amplified whereas high strains are dampened [122,180]. Chondrocytes deform almost instantaneously under load and recover their shape
gradually when the load is removed [178]. Therefore, during cyclic loading, the deformation of chondrocytes is not as great during each individual cycle [155, 178, 181].

It is known that mechanical loading is linked to an adaptation of cartilage properties, as the load-bearing areas are richer in PGs and thicker than the adjacent non-load-bearing regions [182, 183]. Furthermore, chondrocytes and their intracellular organelles tend to be larger in the more weight-bearing locations [184]. Further support for the importance of mechanical loading can be seen from changes that occur due to a lack of loading, as joint immobilization causes PG depletion, cartilage thinning and decreased PG synthesis [185, 186]. In addition, chondrocytes are sensitive to the type of loading applied, i.e., static loading suppresses GAG synthesis in a magnitude-dependent manner [187, 188], whereas moderate dynamic loading increases GAG synthesis [174, 175]. For example, 0.01 – 1 Hz oscillatory compression of cartilage explants with 1 – 5% strain stimulated GAG synthesis, whereas a lower frequency (< 0.001 Hz) loading had no effect on GAG synthesis [188]. At the same time, an excessively high magnitude or too high frequency loading has been reported to decrease GAG synthesis [174–176]. Tissue engineered cartilage constructs also respond in a similar way to static and dynamic loading as these native plugs of cartilage [189]. Thus, it has been suggested that the streaming potentials caused by dynamic loading exert beneficial effects on cartilage homeostasis, whereas the hydrostatic pressure attributable to static loading is detrimental [190].

Cellular deformation due to mechanical loading is one of the proposed pathways exploited by chondrocytes to perceive changes in their environment [191]. One simple pathway for this is through activation of stretch-sensitive ion channels, i.e., the compression of cartilage will flatten chondrocytes and cause membrane stretch [192]. Activation of these channels will allow ion flux through the cell membrane leading to changes in intracellular ion concentrations and subsequent modulation of cell synthesis [193]. In addition to physical compression of the cell, chondrocytes are sensitive to extracellular osmolarity and modulate their shape when there are changes in matrix hydration [194]. Compared to many other cells, the resting membrane potential of chondrocytes is not very negative (~ −10 mV); this has been suggested to protect chondrocytes from osmotic challenge [195]. This proposal was based on the observation that chondrocytes clamped to −80 mV (compared to their natural −10 mV condition) swell substantially when extracellular osmolarity is decreased and cannot shrink back to their original volume when the initial higher osmolarity is restored [195].

The regulation of volume is known to be vital for chondrocytes and occurs in health and disease [196]. Chondrocytes in situ exhibit a regulatory volume decrease (RVD) when exposed to a hypo-tonic challenge, whereas they have only a limited capacity to increase their volume in response to a hyper-osmotic challenge [197, 198]. Under iso-osmotic conditions, the water content of chondrocytes is approximately 60% [170, 199]. Chondrocytes also adapt to loading conditions as non-strenuous exercise has been shown to increase the cell volume of rabbit cartilage chondrocytes [200]. Due to cartilage degeneration, both the water content and the permeability of cartilage become increased, which exposes chondrocytes to more varying osmotic conditions during tissue compression [201]. Increased ECM hydration also causes cell swelling as chondrocytes are permeable to water. However, it has been observed that chondrocytes within degenerating cartilage swell more than predicted by only
the increased hydration [153]. This swelling could be due to an increase in the size of their intracellular organelles, an increased intracellular osmolyte concentration or reduced RVD capacity [153]. Whatever is the case, swelling is thought to be an unfavorable event since swollen chondrocytes are more likely to be injured due to impact loading [202]. It has been also observed that the chondrocyte cell membrane contains numerous ruffles and the area of cell membrane can more than double before cell lysis occurs [170]. The ruffles in the membrane can be seen as an excess membrane area, allowing large deformations without substantial membrane stress [170].

**3.2 CHARACTERIZATION OF CELLS AND CARTILAGE PROPERTIES**

If one wishes to predict how chondrocytes perceive their surrounding environment, then one must be able to perform an accurate quantification of tissue and cell properties. It is important to analyze living chondrocytes in their native environment as the cells will behave differently in their microenvironment as compared to their characteristics in cell culture or other in vitro conditions [24, 31, 32, 94, 108, 117, 203]. Confocal laser scanning microscopy (CLSM) has become a popular method for studying living chondrocytes in situ, or even in vivo [24, 155, 204]. CLSM is a technique that utilizes fluorescence light, and therefore the sample must contain components that are intrinsically fluorescent or the sample needs to be stained with a fluorescent dye that becomes attached to the structures to be visualized. The fluorescein molecules, fluorophores, absorb light at certain wavelength and emit it back at a longer wavelength, which can then be detected. Because laser illumination and light emission are limited to a small focal spot by two ‘confocal’ pinholes, it is possible to discard out-of-focus light throughout the depth of the specimen [205]. This allows scanning of optical sections at different depths that can be combined to create three dimensional data [204]. The resolution of CLSM is in the sub-micrometer scale, which allows good quantification of cells, surrounding structures and cellular processes [204–206]. A similar operational principle is utilized in dual-photon excitation (DPE) microscopy (also referred to as multi-photon microscopy (MPM)), where two photons of smaller energy are simultaneously absorbed by the fluorophore [155, 207]. This leads to less photo-toxicity and reduced photo-bleaching effects, as only fluorophores in the focal spot are excited, as compared to the traditional one-photon CLSM [207, 208]. The disadvantage in the DPE microscopy is that more powerful (expensive) lasers are needed to generate sufficient photon flux to excite the fluorophores. Live cells from translucent samples can also be visualized using transmitted light microscopy, such as phase contrast and differential interference contrast microscopy [209]. Unfortunately, these methods cannot be applied to thick samples.

Cartilage structure and composition can be quantified by microscopic, spectroscopic and biochemical methods [210–214] and, to a certain extent, with traditional imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound [215–218]. The cartilage collagen network has been traditionally visualized from histological samples using polarized light microscopy (PLM) [47], and modern equipment with rotating polarizer-analyzer pair allows the calculation of an average collagen orientation in each imaging pixel [214, 219]. PLM can be considered to be the gold standard in the
quantification of fibril orientation, but it requires histological sections, and hence, the destruction of the sampled cartilage. More recent methods, including at least MRI, polarization-sensitive optical coherence tomography (OCT) and MPM, have made possible the in vivo characterization of the collagen network orientation [220, 221]. Furthermore, OCT and MPM can be performed during arthroscopy, whereas MRI is totally non-invasive (but is the least accurate technique) [220]. In addition, other methods that require sample preparation are available, such as those based on X-ray diffraction and scattering [46,222].

Biochemical and radiochemical methods have been traditionally used for assessing the proteoglycan and collagen content in the articular cartilage, and these could be considered as gold standard methods [210,223–225]. The collagen content can be indirectly approximated by assaying the hydroxyproline content of the sample, whereas the proteoglycan content can be approximated from hexosamine and uronic acid concentrations, or by measuring the fixed charge density (FCD) of the sample [223, 226, 227]. FCD can be calculated by measuring the streaming potentials that arise when an electrolyte solution flows through cartilage due to the pressure gradient, or more easily with a tracer method, such as one in which cartilage is equilibrated in a dilute electrolyte solution and the uptake of cations is measured [225, 225]. These methods, however, provide only a bulk value for the tissue under inspection and thus, require cutting of multiple cartilage slices in order to obtain depth-dependent information about the composition of the tissue. One viable option to overcome this limitation is to use safranin O staining and to assess the dye concentration, as safranin O binds stoichiometrically to GAG polyanions, i.e., one molecule of dye binds to each negatively charged GAG group [228]. The dye content can be assessed with digital densitometry (DD); i.e., the light absorbance due to safranin O staining is measured and related to the dye content via the Beer-Lambert law [228,229].

In addition to these biochemical methods [210,230], the properties of cartilage can be quantified with vibrational spectroscopic techniques. Fourier transform infrared (FTIR), near infrared (NIR) and Raman spectroscopy are all suited for this purpose [231]. FTIR and Raman can be combined with microscopy to image histological sections with high molecular specificity, whereas NIR offers better tissue penetration; for example, NIR could be used during arthroscopic surgery [232]. In each method, the underlying signal originates from the vibrational energy levels associated with chemical bonds in the sample. In Raman spectroscopy, the sample is illuminated with a laser and inelastic scattering of light, which causes a shift in the energy of the laser photons, is recorded. The change in wavelength is called the Raman shift and depends on the underlying chemical bond [211]. In FTIR and NIR, the sample is illuminated with infrared light and the amount of absorbance (or reflectance) at different wavelengths is recorded. As chemical bonds only absorb energy at certain frequencies, the amount of absorbance can be related to the chemical composition [231]. The first studies on cartilage using FTIR were performed with pure compound mixtures of collagen and aggrecan [213,233]. In these works, it was shown that absorbance in the Amide I (1584 - 1720 cm⁻¹) and carbohydrate (984-1140 cm⁻¹) regions correlated with the amount of collagen and aggrecan, respectively. These are univariate parameters, as only a part of the spectrum (absorbance against wavelength) is used to approximate the sample composition. In addition, multivariate methods have been proposed, where different parts of the spectrum are simultaneously studied, or even the whole spectrum can be utilized [211,231,234,235].
4 Material models of articular cartilage

The most widely used material model of articular cartilage is based on the biphasic mixture theory [41,236,237]. The model is isotropic and divides cartilage into two phases, solid collagen-PG mesh and interstitial fluid, which both are assumed to be incompressible [41,236,237]. The model can replicate the response of cartilage in confined compression, but can not capture tensile behavior or predict what occurs in unconfined compression [41,43,238,239]. A natural extension to the biphasic model is the triphasic theory, which introduces an additional ion phase to explain the swelling behavior of cartilage [5]. The ion phase can be further divided into anions and cations, which leads to the quadriphasic model of cartilage [240]. These models, however, do not take into account the anisotropy of cartilage. Hence, in attempts to more realistically capture the structure, cartilage has been modeled as transversely isotropic [43], cone-wise linear elastic [87], biphasic poroviscoelastic [241] and fibril-reinforced biphasic with elastic or viscoelastic fibrils [242,243]. These models can successfully predict the tissue response due to swelling or due to compression in unconfined, confined or indentation geometries, but not simultaneously in all configurations. When tissue swelling caused by proteoglycan FCD is modeled simultaneously with an anisotropic, viscoelastic collagen network, it is possible to model all these experiments with a single set of material parameters [244]. This leads to the fibril-reinforced poroviscoelastic swelling material model of cartilage [244]. Fibril-reinforcement can be modeled in several ways, and some formulations that take account of anisotropic and strain-dependent permeability have also been proposed [245–248].

4.1 POROELASTIC BIPHASIC MODEL

The linear poroelastic biphasic theory was first introduced for analysis of wet soils by Biot in 1941 [249]. The governing equations in the biphasic theory and in the poroelastic theory are mathematically equivalent [76,250]. Because of its longer history, poroelastic properties can be readily applied in many commercial finite element software through soil mechanics [251,252]. The governing equation for total stress in a poroelastic biphasic material ($\sigma_{\text{tot}}$) can be written as

$$\sigma_{\text{tot}} = \sigma_s + \sigma_{\text{fl}}, \quad (4.1)$$

where $\sigma_s$ and $\sigma_{\text{fl}}$ are the solid and fluid stress tensors, respectively. Further, these are defined as

$$\sigma_s = -n_s p \mathbf{1} + \sigma_{\text{eff}}, \quad (4.2)$$
$$\sigma_{\text{fl}} = -n_f p \mathbf{1}, \quad (4.3)$$

where $n_s$ is the solid volume fraction, $n_f$ is the fluid volume fraction, $p$ is the fluid pressure, $\mathbf{1}$ is the unit tensor and $\sigma_{\text{eff}}$ is the effective solid matrix stress. If we insert equations (4.2) and (4.3) into equation (4.1), and take into account that $n_f + n_s = 1$, then the total stress becomes
By applying the Voigt notation for the stress and strain tensors, the equation (4.5)
\[
\sigma_{\text{tot}} = -pI + \sigma_{\text{eff}}.
\] (4.4)
Hence, the effective stress tensor alone resists deformation if there is no fluid flow. For linear elastic materials, the effective stress is mapped to strain (\(\epsilon\)) through a fourth-order stiffness tensor \(C\) as [253]
\[
\sigma_{\text{eff}} = C : \epsilon.
\] (4.5)
By applying the Voigt notation for the stress and strain tensors, the equation (4.5) can be presented in a matrix form as
\[
\{\sigma_{\text{eff}}\} = [C] \{\epsilon\}.
\] (4.6)
In the most simple case of a linear isotropic elastic material, the stress-strain relation takes the form
\[
\begin{bmatrix}
\sigma_{\text{eff11}} \\
\sigma_{\text{eff22}} \\
\sigma_{\text{eff33}} \\
\sigma_{\text{eff23}} \\
\sigma_{\text{eff12}}
\end{bmatrix} = \frac{E}{(1+\nu)(1-2\nu)} \begin{bmatrix}
1-\nu & \nu & \nu & 0 & 0 & 0 \\
\nu & 1-\nu & \nu & 0 & 0 & 0 \\
\nu & \nu & 1-\nu & 0 & 0 & 0 \\
0 & 0 & 0 & 1-2\nu & 0 & 0 \\
0 & 0 & 0 & 0 & 1-2\nu & 0 \\
0 & 0 & 0 & 0 & 0 & 1-2\nu
\end{bmatrix} \begin{bmatrix}
\epsilon_{11} \\
\epsilon_{22} \\
\epsilon_{33} \\
\epsilon_{23} \\
\epsilon_{12}
\end{bmatrix},
\] (4.7)
where \(E\) is the linear Young’s modulus and \(\nu\) is the Poisson’s ratio. The linear elastic model assumes, in addition to a linear stress-strain relationship, that deformations are small. Because of this, the use of a hyperelastic material is much more appropriate, as it allows for a non-linear stress-strain relationship that depends on the strain energy density function \(W\). The effective (Cauchy) stress for a hyperelastic material can be given in the form
\[
\sigma_{\text{eff}} = \frac{2}{J} F \left[ \frac{\partial W}{\partial C} (C) \right] F^T,
\] (4.8)
where \(C = F^TF\) is the right Cauchy-Green deformation tensor, \(W = \hat{W}(C)\) is the elastic strain energy and \(J\) is the determinant of the deformation gradient tensor \(F\).

The fluid flow through a solid is due to the pressure gradient \(\nabla p\) and, in the general case, the magnitude of the fluid flow rate \(q = [q_x, q_y, q_z]^T\) is controlled by the hydraulic permeability tensor \(k\) according to Darcy’s law [254]
\[
q = -k \nabla p.
\] (4.9)
If anisotropy of permeability is neglected, the permeability tensor \(k\) can be replaced with scalar permeability \(k\) and the equation for \(q\) is given as
\[
q = -k \nabla p.
\] (4.10)
In addition, the permeability of cartilage is not constant, as during compression, the tissue becomes compacted while fluid flows out. This decreases the permeability because the apparent pore size in the matrix becomes smaller [76]. Thus, the permeability is strain-dependent and can be modeled as [255,256]
by applying the Voigt notation for the stress and strain tensors, the equation (4.5)

\[ k = k_0 \left( \frac{1 + e}{1 + e_0} \right)^M = k_0 J^M, \quad (4.11) \]

where \( k_0 \) is the initial permeability, \( e_0 \) and \( e \) are the initial and current void-ratios, respectively, and \( M \) is a positive material constant. The void ratio is defined as

\[ e = \frac{n_f}{n_s}. \quad (4.12) \]

### 4.2 FIBRIL-REINFORCED SWELLING MODEL

In the fibril-reinforced swelling model, the solid phase is divided into a fibrillar collagen network and a non-fibrillar PG mesh that impedes water and causes tissue swelling [244]. The total stress tensor in the model (\( \sigma_{\text{tot}} \)) can be divided between these two parts of the solid matrix, and to stresses induced by swelling [244,257]

\[ \sigma_{\text{tot}} = \sigma_{nf} + \sigma_f - \Delta \pi I - \mu_1 I - T_c I. \quad (4.13) \]

Here, \( \sigma_{nf} \) and \( \sigma_f \) are the non-fibrillar and fibrillar matrix stresses, respectively, \( I \) is the unit tensor, \( \Delta \pi \) is the Donnan swelling pressure gradient, \( \mu_1 \) is the water chemical potential, and \( T_c \) is the chemical expansion stress.

#### 4.2.1 Non-fibrillar matrix

The compressible non-fibrillar matrix can be modeled using a compressible neo-Hookean material model [94,244,258,259]

\[ \sigma_{nf} = K \frac{\ln(J)}{J} I + \frac{G}{J} (F \cdot F^T - J^2/3 I), \quad (4.14) \]

where \( K \) is the shear modulus, \( G \) is the bulk modulus and \( J \) is the determinant of the deformation gradient tensor \( F \). The bulk and shear moduli are further defined by the non-fibrillar matrix modulus \( E_{nf} \) and Poisson’s ratio \( \nu_{nf} \) as

\[ K = \frac{E_{nf}}{3(1 - 2\nu_{nf})}, \quad (4.15) \]

\[ G = \frac{E_{nf}}{2(1 + \nu_{nf})}. \quad (4.16) \]

The fluid flow in non-fibrillar matrix obeys Darcy’s law with strain-dependency according to the equations (4.10) and (4.11).

#### 4.2.2 Fibril-reinforcement

The fibrillar matrix is constructed with vector-based fibrils that have a distinct initial orientation \( \vec{e}_{f,0} \) and only resist tensile forces [242,243,260]. This approach fundamentally allows the tissue properties to have a complicated directional dependency and different behaviors during compression and tension [29]. The alterations to fibril orientation due to tissue deformation are given as

\[ \vec{e}_f = \frac{F \vec{e}_{f,0}}{\|F \vec{e}_{f,0}\|}, \quad (4.17) \]
where $\vec{e}_f$ is the new fibril orientation. The simplest stress behavior for the fibrils is linear elastic, for which the fibril stress $\sigma_f$ is calculated as

$$
\sigma_f = \begin{cases} 
E_f \varepsilon_f, & \varepsilon_f \geq 0 \\
0, & \varepsilon_f < 0 
\end{cases}
$$

(4.18)

Here $E_f$ is the collagen fibril modulus and $\varepsilon_f = \ln ||\bar{F}_{f,0}||$ is the logarithmic fibril strain. Fibrils can also be modeled as being either viscoelastic or nonlinear, which results in an additional viscoelastic damping coefficient or strain-dependent moduli [244, 260, 261]. In addition to the oriented primary fibril network, the fibril-reinforcement can include randomly oriented secondary fibrils that mimic the collagen cross-links and the apparently random orientation of the fibrils observed under high magnifications [262]. The stresses of primary and secondary fibrils ($\sigma_{f,p}$ and $\sigma_{f,s}$, respectively) are

$$
\sigma_{f,p} = \rho_z C \sigma_f, 
$$

(4.19)

$$
\sigma_{f,s} = \rho_z \sigma_f, 
$$

(4.20)

where $C$ is the volumetric fraction between primary and secondary fibrils and $\rho_z$ is the depth-dependent fibril volume fraction, which represents the cartilage collagen content [29, 244, 258]. The total stress tensor of the fibrillar matrix is given as the sum

$$
\sigma_f = \sum_{i=1}^{N_{f,tot}} \sigma_{f,i},
$$

(4.21)

where $N_{f,tot}$ is the total number of fibrils and $\sigma_{f,i}$ is the stress tensor in each individual fibril.

### 4.2.3 Swelling and electrostatic repulsion

Cartilage PGs are highly negatively charged; they attract positive cations into the interstitial fluid of cartilage [201, 240]. This excess of cations with respect to synovial fluid creates a Donnan swelling pressure gradient $\Delta \pi$ that subsequently allows cartilage to swell. The magnitude of $\Delta \pi$ can be calculated at equilibrium as [240, 263]

$$
\Delta \pi = \phi_{int} RT \left( \sqrt{c_F^2 + 4 \left( \frac{\gamma_{ext}}{\gamma_{int}} \right)^2 c_{ext}^2} - 2 \phi_{ext} c_{ext} RT \right),
$$

(4.22)

where $c_F$ is the fixed charge density, $c_{ext}$ is the external salt concentration (0.15 M), $R$ is the molar gas constant (8.3145 J mol$^{-1}$ K$^{-1}$), $T$ is the absolute temperature (293 K), $\gamma_{int}$ and $\gamma_{ext}$ are the internal and external activity coefficients, and $\phi_{int}$ and $\phi_{ext}$ are the internal and external osmotic coefficients, respectively. Out of these parameters, only the fixed charge density $c_F$ is significantly altered during cartilage loading, and this can be calculated as [257]

$$
c_F = c_{F,0} \left( \frac{n_{f,0}}{n_{f,0} - 1 + f} \right),
$$

(4.23)

where $c_{F,0}$ is the initial fixed charge density and $n_{f,0}$ is the initial fluid volume fraction. In addition to the Donnan swelling pressure gradient, there is chemical
expansion due to electrostatic repulsion between the negatively charged PGs. The chemical expansion stress $T_c$ can be calculated as [5]

$$T_c = a_0 c_F \exp \left( -\kappa \frac{\gamma^\pm_{\text{ext}}}{\gamma^\pm_{\text{int}}} \sqrt{c^- (c^- + c_F)} \right),$$  \tag{4.24}

where $\kappa$ and $a_0$ are material constants and $c^-$ is the concentration of mobile anions.
Aims of the current study

It is known that chondrocytes and their activities in articular cartilage are very sensitive to mechanical loading. Thus, previous studies have investigated the deformation behavior of chondrocytes in various setups and conditions, ranging from native cartilage to cell cultures. These studies have indicated that the properties of the ECM and the PCM are important regulators of cell biomechanics. However, the extent of the variation in chondrocyte biomechanics within different anatomical locations in the knee joint is still poorly understood, nor is it known the extent to which cartilage ECM and PCM composition and structure affect the deformation behavior of chondrocytes. Even less is known about whether there are changes in the biomechanical responses of the chondrocyte or in the depth-dependent cartilage properties if knee joint biomechanics are suddenly altered. To gain insight into these matters, we used a partial meniscectomy rabbit model of OA to investigate the alterations occurring in the cartilage ECM and PCM properties, and in the chondrocyte biomechanical responses only 3 days after surgery. In addition to experimental methods, mechanisms behind the cell-tissue interaction were investigated with finite element modeling.

The aims of this thesis can be summarized as follows:

1. To characterize in situ chondrocyte deformations due to cartilage loading in various anatomical regions and to examine their relationships to the structure and composition of articular cartilage.
2. To investigate in a site-dependent manner what occurs 3 days after a partial meniscectomy to both chondrocyte deformations and articular cartilage structure and composition.
3. To use finite element modeling, combined with experimental data, to discover if the effects of partial meniscectomy on cell volume responses could be reliably predicted, and furthermore to determine the role of the ECM and PCM properties in the altered cell deformation behavior.

This study will represent a step towards a more comprehensive understanding of cell biomechanics and to clarifying the intimate links between the properties of the surrounding cartilage matrix. It is anticipated that this information may help us to understand what occurs in the earliest stages of OA, to identify the most important structural properties in cartilage that should be maintained in order to achieve physiologically relevant chondrocyte biomechanical responses. For example, this knowledge may be used to design more physiological tissue-engineered cartilage replacements or to target drug treatment to certain molecules.
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Materials and Methods

The materials and main methods used for the purposes of addressing the aims of this thesis are summarized in Table 6.1. The research performed in this thesis encompasses a total of 4 studies and the overall workflow that conceptually illustrates all of the studies is presented in Figure 6.1.

Table 6.1: Materials and methods used in studies I–IV.

<table>
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*Six regions were included altogether (figure 6.1).

PM = partial meniscectomy, C-L = contra-lateral, CLSM = confocal laser scanning microscopy, PLM = polarized light microscopy, DD = digital densitometry, FTIRI = Fourier transform infrared imaging, PCM = pericellular matrix, FEA = finite element analysis.

6.1 SAMPLE PROCESSING AND PREPARATION

Knee joints of skeletally mature female New Zealand White rabbits (Oryctolagus cuniculus, 13 ± 1 month, n = 15) were investigated in studies I–IV. Ten rabbits were subjected to lateral PM under general anesthesia 3 days prior to sacrifice. Both the surgical (PM group) and non-operated, contra-lateral (C-L group) knee joints were collected. In addition, both knee joints of five rabbits served as a separate control group (CNTRL). The animals were free to move, but not exercised prior to sacrifice.

In the PM procedure, a 20-25 mm incision was made on the lateral side of the knee joint, parallel and anterior to the lateral collateral ligament. The knee joint was then carefully flexed and rotated externally to expose the lateral meniscus. The anterior horn was transected across the depth while avoiding cutting the tibial cartilage. Then the ligament attachments of the anterior meniscal horn were transected and the piece of meniscus was removed from the joint. Finally, the joint capsule and skin were sutured. A mean of 45% (± 10% 95% confidence interval) of the total meniscal surface area was removed from each knee (Figure 6.1).
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</table>

*Six regions were included altogether (figure 6.1). PM = partial meniscectomy, C-L = contra-lateral, CLSM = confocal laser scanning microscopy, PLM = polarized light microscopy, DD = digital densitometry, FTIRI = Fourier transform infrared imaging, PCM = pericellular matrix, FEA = finite element analysis.

6.1 SAMPLE PROCESSING AND PREPARATION

Knee joints of skeletally mature female New Zealand White rabbits (Oryctolagus cuniculus, 13 ± 1 month, n = 15) were investigated in studies I–IV. Ten rabbits were subjected to lateral PM under general anesthesia 3 days prior to sacrifice. Both the surgical (PM group) and non-operated, contra-lateral (C-L group) knee joints were collected. In addition, both knee joints of five rabbits served as a separate control group (CNTRL). The animals were free to move, but not exercised prior to sacrifice. In the PM procedure, a 20-25 mm incision was made on the lateral side of the knee joint, parallel and anterior to the lateral collateral ligament. The knee joint was then carefully flexed and rotated externally to expose the lateral meniscus. The anterior horn was transected across the depth while avoiding cutting the tibial cartilage. Then the ligament attachments of the anterior meniscal horn were transected and the piece of meniscus was removed from the joint. Finally, the joint capsule and skin were sutured. A mean of 45% (±10% 95% confidence interval) of the total meniscal surface area was removed from each knee (Figure 6.1).
A) Rabbit knee joint samples and tested cartilage regions

- i) Tibia with intact menisci
- ii) Tibia after partial meniscectomy
- iii) Patella
- iv) Femoral condyles
- v) Femoral groove

B) Chondrocyte shape and volume changes due to loading with confocal laser scanning microscopy

C) Determination of cartilage composition with microscopy and spectroscopy

D) Multi-scale finite element model of the experiments, implemented with measured composition & structure

Figure 6.1: Overview of the workflow in the studies I–IV. Cell deformations were studied in studies I and III from the regions marked in A) by an 'X', with i) and ii) providing representative examples of an intact and transected tibia after the partial meniscectomy, respectively. (Continued on the following page)
Knee joints were harvested 3 days after surgery and excess non-cartilaginous tissue was removed together with the fully intact patella. The lateral and medial femoral condyles were separated from the femoral groove and excess underlying bone was stripped off from the tibial plateaus, which were then cut into 6 × 4 mm cartilage on bone blocks in order to fit them into the sample holder. In addition, the lateral aspect of the femoral groove had to be separated from the medial aspect to allow positioning of the sample surface so that it was flat relative to the indenter. Prior to CLSM imaging, the ECM of the sample was stained as described previously, for 4–8 hours with 3 kDa molecular weight fluorescein conjugated dextran (excitation and emission 488/500 nm, Invitrogen, Molecular Probes, OR, USA) suspended in Dulbecco’s Modified Eagle’s medium (DMEM, Sigma Aldrich, MO, USA) at a concentration of 4.8 mg/ml [31, 32]. Before the CLSM measurements, excess dextran was removed by rinsing the sample for 15 minutes in DMEM. The sample was then mounted in a sample holder with dental cement and immersed in DMEM during testing. All tissues were examined within 40 hours of sacrifice and kept in DMEM at 4°C until testing. After CLSM, the samples were placed in neutral buffered 10% formalin solution and shipped in separate containers from the University of Calgary to the University of Eastern Finland.

CNTRL group samples were investigated in studies I and II, C-L and PM groups were compared in study III, and all three groups were included in study IV. Surgical procedures and sacrificing were conducted according to guidelines of the Canadian Council on Animal Care and all procedures were approved by the committee on Animal Ethics of the University of Calgary.

6.2 CELL MORPHOLOGY AND MECHANICAL LOADING

A commercial confocal laser scanning microscope (Zeiss LSM 510 Meta, Carl Zeiss Inc., Germany) mounted on a custom-designed indentation system (details in Han et al. [206]) was used to capture 2D image stacks of in situ chondrocytes before and after loading (Figure 6.1; studies I & III). This same system with varying setups has been utilized in several other studies for in situ imaging of chondrocytes [31, 32, 178, 180, 207, 264]. In the system, a flat-ended cylindrical light-transmittable glass indenter (diameter, \( \phi = 2 \text{ mm} \)) was attached to a motorized x-y-stage and the tissue loading was achieved by pushing the sample against the underside of this fixed indenter. Images were captured through the glass indenter by a 40× 0.8 NA 0.17 mm cover-glass-corrected water-immersion objective. The movement of the sample holder was controlled by a piezo actuator and recorded by a displacement transducer. The contact forces were recorded by a load cell underneath the sample holder.
6.2.1 Indentation testing and imaging protocol

The patella and femoral groove were loaded on the lateral aspect and midway between the proximal and distal ends, femoral condyles were loaded on their summits and tibial plateaus were loaded on a flat surface, shifted ~1 mm laterally from the sample center (Figure 6.1). The indentation loading protocol was identical to that used in a previous study [32]. First, the sample was aligned carefully to achieve a flat contact with the target surface and indenter. Then, a small tare load (0.1 – 0.2 MPa) was set to ensure that there was an initial contact between the sample and indenter. First, image stacks were captured at this time point. Then, indentation was performed with a nominal pressure of 2 MPa at an average speed of 10 μm/s. The resulting displacement was maintained for 20 minutes before imaging the sample again. Image stacks, in plane perpendicular to loading, were captured in 0.5 μm increments (focal plane resolution 0.41 x 0.41 μm, 512 x 512 pixels) up to tissue depth of ~60 μm. However, as image quality decreases with tissue penetration, the analyzed cells were superficial and mostly located between cartilage depths of 20 to 30 μm.

In the experiments, groupings of chondrocytes were identified before loading and observed throughout the experiment to ensure that the same cells (n = 10/sample) could be analyzed from the before and after loading image stacks. Altogether 6 locations in 30 knee joints were imaged (N = 180 samples, 10 knees/group). Out of these 180 samples, in 5 samples, either the staining procedure failed or tracking of cells was impossible due to offset between the before and after loading images. This resulted in 80-100 analyzed cells per site in each group.

6.2.2 Confocal image analysis

The system was calibrated with polystyrene microspheres (Polysciences Inc., Warrington, PA, USA) of known diameter (5.93 ± 0.05 μm). The microspheres were inserted in dextran stained agarose gel and imaged with the experimental setup. A correction factor of 0.83 for the z-axis stretching was obtained by dividing the known microsphere diameter with their apparent height [31,32,206].

Image stacks were imported into ImageJ (National Institutes of Health, USA) and 10 chondrocytes that could be tracked from the before and after images were chosen for morphological analysis. As fluorescence intensity diminishes with imaging depth, the 2D image stacks were attenuation corrected [265]. The chondrocytes of interest were then cropped and an optimal threshold for segmentation was calculated from the middle slice of the 2D-cell image stack using Otsu’s method [207,266]. The 3D-reconstruction of the chondrocyte was generated with a code programmed in Python, which utilized Visualization Toolkit 5.2.0 (VTK, Kitware Inc., USA). In the code, a marching cubes algorithm was used to generate a polygonal isosurface of the thresholded chondrocyte. Using VTK, this isosurface was further processed into a triangle mesh, and the volume of the mesh was calculated [267]. By calculating the cell volume in the loaded, V, and unloaded, (V₀), configurations, the change in cell volume was calculated as \( (V - V₀)/V₀ \). Furthermore, the coordinates of the triangle mesh were imported into MATLAB (MathWorks Inc., MA, USA) and the cell dimensions were estimated by fitting an ellipsoid to this data [268]. Chondrocyte height was determined as the dimension parallel to loading, while width and depth were the major and minor
axes of the ellipsoid in the perpendicular plane (Figure 6.1). Changes in cell dimensions were calculated analogously to volume changes.

In addition to the analysis of cell morphology, tissue strains and modulus were calculated. The global nominal axial tissue strain was calculated by dividing the applied compression (obtained from the displacement transducer) by the sample thickness (from histology). The nominal local axial strain was calculated from the distance between paired cells in the loaded \((d)\) and unloaded \((d_0)\) configurations as \((d - d_0)/d_0\). In the determination of transverse ECM strains, four ‘marker’ cells in the same focal plane \((x - y)\) before and after loading were tracked and an engineering strain tensor \([e]_{2 \times 2}\) was formed. The tensor was diagonalized, such that the resulting eigenvalues represent the major and minor principal strain directions (major and minor transverse strains) [24,31,32]. Measured equilibrium modulus \(E_{\text{measured}} = \sigma/\epsilon\) was corrected for indentation geometry using the equation described by Hayes et al. [269]

\[
E = E_{\text{measured}} \left(\frac{\pi a}{2 h \kappa} \left(1 - v^2\right)\right).
\]

In the equation, \(h\) is sample thickness, \(a\) is indenter radius, \(\kappa(a/h, \nu)\) is a scaling factor and \(\nu\) is Poisson’s ratio. The dimension-dependent scaling factor \(\kappa\) was retrieved from an earlier study; in contrast to the original values reported by Hayes et al. [269], this takes into account the impact of the indenter surface friction [270].

### 6.3 HISTOLOGICAL QUANTIFICATION OF CARTILAGE

Histological analyses were performed to assess depth-wise changes in articular cartilage proteoglycan content, collagen content and collagen fibril network orientation at the site of indentation testing. Before cutting sections for microscopy, the formalin fixed samples were decalcified with ethylene diamine tetraacetic acid (EDTA), dehydrated with an ascending alcohol series (50%, 70%, 80%, 95% and 100%), cleared with xylene and embedded in paraffin [219,228,229,271].

#### 6.3.1 Polarized light microscopy

Sections of 5 \(\mu\)m thickness were deparaffinized with xylene and subjected to removal of PGs with hyaluronidase digestion (1000 U/ml, Sigma-Aldrich, MO, USA) [271]. The PLM system consisted of a polarized light microscope (Leitz Ortholux II POL, Leitz Wetzlar, Germany), a CCD camera (Photometrics CH 250/A, Photometrics Inc., AZ, USA), a light source, monochromators, a depolarizer, and a polarizer pair (details in [219]). The automatic pixel-wise calibration and alignment routine was performed before each measurement [219,272]. For each sample, seven images were captured with 6.3\(\times\) objective (pixel size 3.57 \(\mu\)m) and by rotating the polarizer pair in steps of 15° from the \(0°\)-position up to the \(90°\)-position in between each image. Subsequently, an additional image was acquired with \(\frac{\pi}{4}\)-waveplate inserted and polarizers at the \(90°\)-position. This allows calculation of Stokes parameters \(S_0\)–\(S_3\) as

\[
\begin{align*}
S_0 &= I_{0°} + I_{90°}, \\
S_1 &= I_{0°} - I_{90°}, \\
S_2 &= 2I_{45°} - S_0, \\
S_3 &= S_0 - I_{(90° + \frac{\pi}{4}-\text{wp})°}.
\end{align*}
\]

(6.2)
Here $I$ is the light intensity that goes through the system. The polarizer pair angle is given in the subscript ($\varphi$-wp indicates quarter-wave plate inserted). The Stokes parameter $S_0$ designates the total intensity of light, $S_1$ gives the amount of linear polarization, $S_2$ describes the amount of $\pm 45^\circ$ polarization and $S_3$ refers to the amount of left or right circular polarization. Furthermore, with Stokes parameters, the orientation angle of polarization ellipse can be determined from

$$\theta = \frac{1}{2} \arctan \left( \frac{S_2}{S_1} \right). \quad (6.3)$$

In the case of articular cartilage, the emerging light splits into two components due to birefringence of the collagen fibrils; one component is parallel to the collagen fibril axis with the other being perpendicular to it. Because of this, the polarization ellipse angle directly indicates the average collagen fibril orientation in the image pixel as micrometer-scale pixels are still relatively large compared to collagen fibrils [219].

### 6.3.2 Digital densitometry

Sections of 3 µm were deparaffinized with xylene and stained for digital densitometry (DD) by Safranin O staining [32, 228, 229, 273]. Binding of Safranin O to negatively charged articular cartilage glycosaminoglycans (GAGs) is stoichiometric, which means that the staining intensity is an indirect estimate of the tissue PG content obtained by estimating the fixed charge density (FCD) [228, 229]. The Safranin O stained sections were visualized in a light microscope (Nikon Microphot FXA, Nikon PSM-4, Nikon, Japan) and imaged under monochromatic light (wavelength 492 ± 5 nm) with a CCD camera (ORCA-ER, Hamamatsu Photonics, Japan). The camera was calibrated with neutral density filters (Schott, Germany), allowing conversion of image 12-bit grayscale values into absorbance [228, 274]. The ECM was imaged with 4×-objective, resulting in a pixel size of 1.56 µm. A higher magnification (40×-objective) was used to capture the FCD in the PCM of the superficial zone chondrocytes.

### 6.3.3 Fourier transform infrared imaging

Sections of 5 µm thickness were deparaffinized for FTIR spectroscopy. With FTIR imaging, the relative abundance of different molecular constituents can be estimated spatially. Measurements were conducted with a PerkinElmer Spotlight 300 device (PerkinElmer, CO, USA) coupled with a CO2-free dry air purge system (FT-IR purge gas generator, Parker Hannifin Corporation, MA, USA). Samples were placed on KBr windows and measured in the transmittance mode. By using a spatial pixel size of 6.25 µm, spectra were collected from a 150 µm wide region of interest extending from the cartilage surface to the subchondral bone. Data were imported and analyzed with custom code in MATLAB (MathWorks Inc., MA, USA). Each spectrum was baseline corrected by minimum subtraction and then the mean integrated area of the Amide I peak region (1585 – 1720 cm$^{-1}$) was calculated to estimate the collagen content [8, 162, 213, 234, 275–277].

### 6.4 FINITE ELEMENT MODELING

In study IV, axisymmetric, multi-scale finite element (FE) models were constructed for each knee joint location for the PM and CNTRL groups (12 multi-scale models).
Each model included a macro-scale cartilage model (site model) and micro-scale cell model (Figure 6.1). The FE models were created in ABAQUS, using 4-node, axisymmetric, pore pressure elements (CAX4P). Denser meshes were constructed around the indenter edge and for the first 30% of cartilage thickness from the articular surface (Figure 6.1). The geometry of the macro-model was site- and group-specific, as the thickness of the model was taken as the average cartilage thickness determined from the digital densitometry measurements (Table 6.4.2). Cell model geometry was initially the same in each model – a round cell (r = 5.1 µm) with a 1.6 µm thick PCM-layer was generated at a depth of 22 µm from the cartilage surface (Figure 6.1). However, the initial swelling step of the tissue produced slight variations in the cell shape between different models.

### 6.4.1 Implementation of material properties

The cartilage ECM and PCM were modeled using fibril-reinforced, poroelastic, swelling (FRPES) material properties (Section 4.2). Chondrocytes were modeled without the fibril-reinforcement. The indenter was assumed to be rigid in all of the models. Collagen content was taken into account with the fibril volume density, ρz, which was approximated from the FTIR data (Figure 6.2). The collagen orientation in the ECM was implemented based on the PLM data (Figure 6.2), with superficial zone thickness, dvec, and bending radius of the collagen fibrils in the middle zone, rvec, as previously [258,263,278]. The orientation angle in the PCM was assumed to be parallel to the cell surface at all points (Figure 6.1). Fixed charge density (FCD), cF, in the ECM was implemented according to the DD data (Figure 6.2) by converting optical density (OD) values into FCD according to Király et al. [229]

\[
c_F^{ECM} = \frac{OD + 1.105}{15.153} \text{mEq/ml.} \quad (6.4)
\]

Subsequently, FCD in the PCM was implemented as

\[
c_F^{PCM} = \alpha_{PCM} \cdot c_F^{ECM}, \quad (6.5)
\]

where the multiplier \( \alpha_{PCM} \) was calculated from the normalized cell-level DD data (Table 6.3). The non-fibrillar part of the ECM was modeled with non-linear neo-Hookean poro-hyperelastic material properties, with matrix modulus, \( E_{nf} \), Poisson’s ratio, \( \nu_{nf} \), initial permeability, \( k_0 \), and permeability strain-dependency factor, M. Swelling of the material was modeled through osmotic swelling and chemical expansion as described in equations (4.22) and (4.24).

### 6.4.2 Finite element analysis

The experimental indentation protocol, described in section 6.2.1, was simulated using the multi-scale approach. In the simulations, the indenter was moved into contact with the cartilage surface after free swelling of the tissue. An indentation step was performed with a compression rate of 10 µm/s up to experimentally observed tissue strain. Afterwards, the tissue was allowed to relax for 20 minutes, as done in the experiments. This simulation was repeated manually to optimize the material properties of the ECM (Table 6.3), namely collagen stiffness, \( E_l \), non-fibrillar matrix modulus, \( E_{nf} \), and initial permeability, \( k_0 \), until the error between the simulated and experimental peak and equilibrium forces was less than 2%. After optimizing the site model force-responses, the cell models were
simulated. Each cell model was driven by the nodal pore pressures and displacements of the site model. After the simulations, cell volume after loading in equilibrium, $V$, and before loading but after free-swelling, $(V_0)$, were used to calculate cell volume change as $(V - V_0)/V_0$. 

Table 6.2: General mechanical and compositional parameters in the FE models.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ECM</th>
<th>PCM</th>
<th>Chondrocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_f$ (MPa)</td>
<td>$[3.5 – 7.0]\cdot 10% E_{ECM}$</td>
<td>$[0.7 – 1.3]\cdot 10% E_{ECM}$</td>
<td>$3.3% E_{ECM}$</td>
</tr>
<tr>
<td>$k_0$</td>
<td>$[1.3 – 3.5]\cdot 10% k_{ECM}$</td>
<td>$0.79$</td>
<td>$0.3$</td>
</tr>
<tr>
<td>$\nu_{nf}$</td>
<td>$0.42$</td>
<td>$0.3$</td>
<td>$0.3$</td>
</tr>
<tr>
<td>$M$</td>
<td>$8.1$</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$n_f$</td>
<td>$0.85$</td>
<td>$-0.15$</td>
<td>$0.85$</td>
</tr>
<tr>
<td>$\theta_{col}$ (°)</td>
<td>PLM data</td>
<td>Tangential to cell surface</td>
<td>FTIR data</td>
</tr>
<tr>
<td>$\rho_z$</td>
<td>$\rho_{ECM}$</td>
<td>$\rho_{ECM}$</td>
<td>$c_F$</td>
</tr>
<tr>
<td>$\alpha_{PCM} \cdot c_{ECM}$</td>
<td>$90% c_{ECM}$</td>
<td>$90% c_{ECM}$</td>
<td></td>
</tr>
</tbody>
</table>

* Parameter is knee joint location-specific, [minimum-maximum] range is given.

$E_f = $ fibril network modulus, $E_{nf} = $ non-fibrillar matrix modulus, $k_0 = $ initial permeability, $\nu_{nf} = $ Poisson's ratio, $M = $ permeability strain-dependency factor, $n_f = $ fluid fraction, $\theta_{col} = $ collagen orientation, $\rho_z = $ collagen fibril density, $c_F = $ fixed charge density.

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simulated. Each cell model was driven by the nodal pore pressures and displacements of the site model. After the simulations, cell volume after loading in equilibrium, $V$, and before loading but after free-swelling, $(V_0)$, were used to calculate cell volume change as $(V - V_0)/V_0$.

Table 6.2: General mechanical and compositional parameters in the FE models. One order of magnitude difference between the PCM and ECM properties was implemented [103, 112]. Chondrocyte properties were taken from earlier studies [170, 279, 280]. Fluid volume fraction was implemented as a function of normalized cartilage depth $z$ (0 = cartilage surface, 1 = cartilage-bone interface) [257].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ECM</th>
<th>PCM</th>
<th>Chondrocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_f$ (MPa)</td>
<td>[3.5 – 7.0]*</td>
<td>$10% \cdot E_f^{ECM}$</td>
<td>–</td>
</tr>
<tr>
<td>$E_{nf}$ (MPa)</td>
<td>[0.7 – 1.3]*</td>
<td>$10% \cdot E_{nf}^{ECM}$</td>
<td>$3.3% \cdot E_{nf}^{ECM}$</td>
</tr>
<tr>
<td>$k_0$ ($10^{-15}$ m$^4$ N$^{-1}$)</td>
<td>[1.3 – 3.5]*</td>
<td>$10% \cdot k_0^{ECM}$</td>
<td>0.79</td>
</tr>
<tr>
<td>$\nu_{nf}$</td>
<td>0.42</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>$M$</td>
<td>8.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$n_f$</td>
<td>0.85 – 0.15$z$</td>
<td>0.85</td>
<td>0.6</td>
</tr>
<tr>
<td>$\theta_{col}$ ($^\circ$)</td>
<td>PLM data</td>
<td>Tangential to cell surface</td>
<td>–</td>
</tr>
<tr>
<td>$\rho_z$</td>
<td>FTIR data</td>
<td>$\rho_z^{ECM}$</td>
<td>–</td>
</tr>
<tr>
<td>$c_F$</td>
<td>DD data</td>
<td>$\alpha_{PCM} \cdot c_F^{ECM}$</td>
<td>$90% \cdot c_F^{ECM}$</td>
</tr>
</tbody>
</table>

* Parameter is knee joint location-specific, [minimum-maximum] range is given.

$E_f$ = fibril network modulus, $E_{nf}$ = non-fibrillar matrix modulus, $k_0$ = initial permeability, $\nu_{nf}$ = Poisson’s ratio, $M$ = permeability strain-dependency factor, $n_f$ = fluid fraction, $\theta_{col}$ = collagen orientation, $\rho_z$ = collagen fibril density, $c_F$ = fixed charge density.

Table 6.3: Group and site-specific mechanical and compositional parameters in the FE models.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patella</th>
<th>Femoral Groove</th>
<th>Lateral Femur</th>
<th>Lateral Tibia</th>
<th>Medial Femur</th>
<th>Medial Tibia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimized</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_f$ (MPa)</td>
<td></td>
<td>7.0</td>
<td>4.0</td>
<td>6.0</td>
<td>7.0</td>
<td>4.0</td>
</tr>
<tr>
<td>$E_{nf}$ (MPa)</td>
<td></td>
<td>0.7</td>
<td>1.3</td>
<td>0.8</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>$k_0$ ($10^{-15}$ m$^4$ N$^{-1}$)</td>
<td></td>
<td>2.0</td>
<td>1.3</td>
<td>3.5</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>$\alpha_{PCM}$</td>
<td></td>
<td>1.119</td>
<td>1.063</td>
<td>1.012</td>
<td>1.044</td>
<td>1.078</td>
</tr>
<tr>
<td>$h$</td>
<td></td>
<td>877 $\mu$m</td>
<td>538 $\mu$m</td>
<td>472 $\mu$m</td>
<td>388 $\mu$m</td>
<td>559 $\mu$m</td>
</tr>
<tr>
<td>CNTRL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_{PCM}$</td>
<td></td>
<td>1.231</td>
<td>1.142</td>
<td>1.078</td>
<td>1.077</td>
<td>1.170</td>
</tr>
<tr>
<td>$h$</td>
<td></td>
<td>831 $\mu$m</td>
<td>534 $\mu$m</td>
<td>421 $\mu$m</td>
<td>384 $\mu$m</td>
<td>589 $\mu$m</td>
</tr>
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<td>PM</td>
<td></td>
<td></td>
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<tr>
<td>$\alpha_{PCM}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$h$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$E_f$ = fibril network modulus, $E_{nf}$ = non-fibrillar matrix modulus, $k_0$ = initial permeability, $\alpha_{vec}$ = superficial zone thickness, $r_{vec}$ = bending radius of middle zone fibrils, $\alpha_{PCM}$ = PCM to ECM fixed charge density, $h$ = cartilage thickness.
Figure 6.2: Measured depth-wise properties of cartilage were implemented into the FE model. Collagen orientation was implemented according to PLM-derived SZ and MZ percentage thicknesses, as done previously [258,263,278]. FCD distributions were implemented by fitting 12th order polynomials to the data. Collagen content was implemented with linear fit to FTIR spectroscopic data, similarly as done previously [261]. The average profiles shown are from the lateral femoral cartilages.
6.5 STATISTICAL ANALYSIS

In all statistical tests, the critical-level of significance was set to $\alpha = 0.05$.

Study I: Cell volume and dimensional data (values before loading, after loading and the change due to loading) were pooled for each knee joint location and data normality was analyzed with the Kolmogorov-Smirnov test ($n > 50$); strain data (global, local axial and local transverse strains), Young’s moduli and compositional data were also pooled for each location and data normality was assessed using the Shapiro-Wilk test ($n < 50$) [281–283]. Analysis of variance (ANOVA) with Tukey’s post hoc follow-up test was used to test the significance of parameter differences between the knee joint locations. Finally, mean parameter values for each location were calculated and Pearson correlations coefficients were determined between changes in cell morphology (cell volume, height, depth and width changes) and tissue strains; or between changes in cell morphology and cartilage composition/structure. Analyses were performed using IBM SPSS Statistics 19.0 (IBM Corp, NY, USA).

Study II: Depth-wise PG content (ECM- and PCM-level), collagen content and collagen orientation profiles were pooled by knee joint location. Comparisons were made between contacting knee joint locations, and data were tested for normality using Shapiro-Wilk test. If normality could be assured, independent t-tests were performed. In the case that normality could not be assured (e.g., for each point of the depth-wise profiles), comparisons were made with the Mann-Whitney U-test. Multivariable linear regression analysis was performed after pooling observations from all knee joint locations. Cell morphology changes were set as dependent variables, and PG content, collagen content and collagen orientation were designated as predictors. The depth-dependency of cartilage composition was taken into account by building regression models across tissue depths from 0 to 100 µm. Analyses were performed using IBM SPSS Statistics 19.0 (IBM Corp, NY, USA).

Study III: Initial cell morphology, changes in cell morphology and superficial composition of cartilage (average value up to 10% of depth) were compared between C-L and PM knee joint tissues. To test if the C-L group would be appropriate to use as a control group, a Schuirmann’s two one-sided tests was used to investigate equivalence between the C-L group and CNTRL group (from study I) cell volumes. Since the difference was less than ±5%, this was concluded to be sufficient for our purposes. Comparisons were performed for each knee joint locations using independent samples t-tests. Analyses were performed using IBM SPSS Statistics 21.0 (IBM Corp, NY, USA).

Study IV: Linear mixed models (LMMs) were used to test the significance of point-by-point differences in composition between the groups. With LMM, potential interrelationship between samples from the same animal can be taken into account [273,284–286]. Analyses were conducted separately for each knee joint location. In all analyses, the sample group (CNTRL, C-L or PM) was set as a fixed factor. At the ECM-level composition analysis, animal (as a categorical variable, subject) was coded as a random variable, whereas in the PCM-level analysis, the analyzed section was coded as an animal specific random variable (multiple cells from same section). Restricted maximum likelihood (REML) estimation was used to calculate marginal means for groups and Bonferroni adjusted 95% confidence intervals. All statistics were carried out in IBM SPSS Statistics 21.0 (IBM Corp, NY, USA).
Results

The following chapter summarizes the most important findings in the studies I-IV.

7.1 REGIONAL CELL-TISSUE INTERACTIONS IN HEALTHY RABBIT KNEE JOINT CARTILAGE

Control group cell responses and articular cartilage composition were investigated in studies I and II. The shape of the superficial zone chondrocytes (i.e., cell height, depth and width) and cell volume varied significantly before loading in the six studied knee joint locations. Prior to loading, the lowest mean cell volume of 288 µm³ was observed in medial tibia, whereas the mean cell volume was between 474 µm³ and 612 µm³ in patella, femoral groove, femoral condyles and lateral tibia. The location-specific average cell height varied from 5.8 to 6.6 µm, cell depth varied from 8.6 to 12.3 µm, and cell width ranged between 10.6 and 17.1 µm. Loading caused a decrease in the average cell volume and cell height at all locations, whereas the average cell width and depth increased (Figure 7.1).

Figure 7.1: Changes in cell volume, height, width and depth due to cartilage indentation loading were highly site-specific in the CNTRL group, even though loading protocol was same at each location. For each parameter, the knee joint locations that do not share a common letter are significantly (*p* < 0.05) different from each other (ANOVA followed by Tukey Post hoc test). As 10 cells were analyzed per sample, the resulting sample size was *n* = 80–100 chondrocytes/location. Means ± 95% confidence intervals are shown.

The mean axial tissue strain due to loading was lowest in the lateral tibial cartilage (9.8 ± 1.6%) and highest in the medial tibial cartilage (22.4 ± 3.2%). The local axial tissue strain was higher than the global tissue strain in each studied knee joint region, ranging from 20.7% to 35.0%. The reduction in cell height due to...
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Chondrocyte shape changes due to loading

![Figure 7.1](image)

**Figure 7.1:** Changes in cell volume, height, width and depth due to cartilage indentation loading were highly site-specific in the CNTRL group, even though loading protocol was same at each location. For each parameter, the knee joint locations that do not share a common letter are significantly (p < 0.05) different from each other (ANOVA followed by Tukey Post hoc test). As 10 cells were analyzed per sample, the resulting sample size was n = 80 – 100 chondrocytes/location. Means ± 95% confidence intervals are shown.

The mean axial tissue strain due to loading was lowest in the lateral tibial cartilage (9.8 ± 1.6%) and highest in the medial tibial cartilage (22.4 ± 3.2%). The local axial tissue strain was higher than the global tissue strain in each studied knee joint region, ranging from 20.7% to 35.0%. The reduction in cell height due to
compression was observed lie between the global and local axial strain magnitudes. Average major and minor transverse tissue strains were lower as compared to axial strain and varied from 4.7% to 10.9% and from 2.6% to 5.9%, respectively. Pearson correlation analysis was performed after pooling the data for each knee joint location (n = 6). Evidently, the axial tissue strain was associated with the decrease in the cell height (r = 0.99, p < 0.001) and the major transverse strain was correlated with the increase in the cell width (r = 0.96, p = 0.002). The minor transverse strain was more strongly correlated to the cell depth increase than the major strain (r = 0.822, p = 0.045, compared to r = 0.516, p = 0.295).

**Structure, composition, and relation to cell deformations**

In study II, cartilage composition and structure was thoroughly measured in a depth-wise manner and the correlation to chondrocyte deformations was investigated using multivariable regression. Correlations were strongest when the cartilage composition were calculated from a depth of approximately 15 to 30 μm, which corresponds well to the assumed depth of CLSM imaging in study I. In the final regression models (Table 7.1), the average matrix content was calculated from 15 to 30 μm of cartilage depth (Figure 7.2). In this very superficial layer, the mean PG content was 40% lower (p < 0.01) and mean collagen orientation angle was 40% higher (p < 0.05) in patellar cartilage as compared to the femoral groove (p < 0.01). In addition, the mean collagen content was 22% lower (p < 0.05) and the mean collagen orientation angle was 44% greater in lateral femoral cartilage as compared to the lateral tibial cartilage (Figure 7.2). The medial tibial and femoral cartilages did not display any significant difference in composition at this depth. Note that only comparisons between contacting anatomical regions were considered.

![Figure 7.2: Average cartilage composition calculated from 15 to 30 μm of tissue depth. Presented values are (mean ± 95% confidence intervals). Comparisons are performed with independent samples t-test.](image_url)
Multivariable regression models were built by considering the cell width, height and volume changes due to indentation as dependent variables, whereas collagen orientation, proteoglycan content and collagen content were set as predictors (independent variables). The parameter estimates are summarized in Table 7.1.

Table 7.1: Parameter estimates for the linear multivariable regression models. Mean values and 95% confidence intervals (CI) are reported for the dependent variables and predictors \((n = 57)\). Unstandardized regression coefficients \((B)\) indicate how much absolute changes in predictor will affect the dependent variable, whereas standardized regression coefficients \((\beta)\) describe relative strength between the variables by considering how many standard deviations the dependent variable will change when predictor value increases by one standard deviation.

<table>
<thead>
<tr>
<th>Dependent variable (mean ± 95% CI)</th>
<th>Predictors (mean ± 95% CI), unstandardized ((B)) &amp; standardized ((\beta)) regression coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intercept</td>
</tr>
<tr>
<td>Cell width change ((7.4 ± 1.7))%</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.001)</td>
</tr>
<tr>
<td>Cell height change ((-19.6 ± 2.1))%</td>
<td>-3.18</td>
</tr>
<tr>
<td></td>
<td>(p = 0.048)</td>
</tr>
<tr>
<td>Cell volume change ((-9.4 ± 1.8))%</td>
<td>-2.71</td>
</tr>
<tr>
<td></td>
<td>(p = 0.978)</td>
</tr>
</tbody>
</table>

\(^a\) PLM = polarized light microscopy, DD = digital densitometry, FTIR = Fourier transform infrared imaging, OD = optical density, ABS = absorbance.

According to the regression analysis, samples with greater collagen orientation angles exhibited larger changes in the chondrocyte width \((\beta = 0.40, p < 0.001)\) and a greater reduction in the cell height \((\beta = −0.25, p = 0.048)\) due to the cartilage compression. Samples with a higher proteoglycan content underwent a smaller cell volume loss \((\beta = 0.28, p = 0.025)\) due to compression. Finally, samples with a higher collagen content exhibited less extensive changes in chondrocyte width \((\beta = −0.40, p < 0.001)\), lower chondrocyte height changes \((\beta = 0.46, p < 0.001)\) and a smaller volume reduction \((\beta = 0.44, p = 0.002)\) due to compression. The goodness of fit is illustrated for each model by plotting predicted values against the observed ones (Figure 7.3).
The variation in pericellular PG content between contacting anatomical regions (Figure 7.4) was rather similar to that observed in the ECM-level analysis (Figure 7.2). On average, the absolute PG content was 19% lower ($p < 0.05$) in the surroundings of patellar chondrocytes as compared to the femoral groove chondrocytes. In addition, the lateral tibial chondrocytes had a reduced amount of PGs in their surroundings, but this difference was limited to approximately 6 μm around the cell; beyond this depth, the difference was no longer statistically significant. The PG content in PCM was $\sim 10\% - 25\%$ higher when compared to the surrounding ECM (Figure 7.4). This normalized PG content was significantly higher in the PCM region of patellar and medial tibial cartilages, when compared to the femoral groove and the medial femoral cartilages, respectively.
7.2 EFFECTS OF PARTIAL MENISCECTOMY ON ARTICULAR CARTILAGE AND CELL DEFORMATIONS

Chondrocyte volume changes were altered after surgery in the PM group when compared to either C-L or CNTRL groups (Figure 7.5). Larger cell volume losses were observed in the PM group at the patellar, femoral groove and lateral femoral cartilage sites, whereas for the medial femoral and medial tibial cartilages, these cell volume losses were reduced in relation to the same sites from either the C-L or CNTRL group. Surprisingly, cell volume changes were similar between all 3 groups at the lateral tibial cartilage site.
**Cartilage composition and structure**

Differences between CNTRL, C-L and PM groups were non-significant ($p > 0.05$) in the depth-wise collagen content and collagen orientation angle profiles in all of the studied anatomical locations. However, a significant ($p < 0.05$) loss of superficial PG content in the PM group was observed in cartilages in all locations, except in the lateral tibial cartilage (Figure 7.6). The extent of this PG loss was mostly limited to the first 20% of cartilage depth, with the exception of medial tibia, where this PG loss extended even further into the depth of the tissue when compared to the C-L group. A similar loss of PG content as in the ECM was observed in the pericellular regions of the chondrocytes (Figure 7.7), with the exception that in the patella sites, there was a significant difference between the C-L and CNTRL groups, whereas C-L and PM groups did not significantly differ from each other.
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Figure 7.6: Depth-wise profiles of proteoglycan content for each studied anatomical site. Mean values with Bonferroni adjusted 95% confidence intervals are presented for each group. Linear mixed models were used to test point-by-point differences between groups, and significant differences ($p < 0.05$) are marked with corresponding dashed lines above the profiles.
Figure 7.7: Cell-level mean PG content profiles through chondrocyte major axis for each studied anatomical location. Linear mixed model was used to test point-by-point significance between the groups and Bonferroni adjusted 95% confidence intervals are given. Significant differences ($p < 0.05$) are marked with corresponding dashed lines.

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7.3 COMPUTATIONAL SIMULATION OF CHONDROCYTE DEFORMATION DUE TO INDEXTATION LOADING

After implementing group- and site-specific cartilage structure and biomechanical properties into the FE models, the experimental indentation protocol was simulated. Chondrocyte volume changes due to the indentation loading were between −5.3% and −19.3% in the simulations, whereas in the experiments, the mean chondrocyte volume changes varied from −1.4% to −19.5% (Figure 7.8). Overall, the magnitudes of cell volume changes were not exactly reproduced, however, the group difference between CNTRL and PM groups at each studied location had a similar trend in the simulations and experiments, e.g., PM group cells underwent larger cell volume changes in comparison to the CNTRL group in patellar, femoral groove and lateral femoral cartilages.

Figure 7.8: Experimental and simulated cell volume changes due to indentation loading at each anatomical location. Cell volume changes predicted by FE modeling were similar to those found experimentally and replicated the trend of site-specific group differences. Mean values and 95% confidence intervals are presented for the experimental data, whereas simulated values are shown with the solid bars.

Parametric analysis

Parametric investigations with different FCD distributions and collagen fibril orientations were performed to assess their effect on the changes in cell volume. The cell volume change was more sensitive to alterations in the PCM FCD as compared to the ECM FCD (Figure 7.9). Interestingly, the absolute cell volume change reached its minimum approximately when the PCM FCD was equal to the chondrocyte FCD. This behavior can be seen in both parametric PCM and ECM + PCM curves. In the latter curve, the ECM FCD was altered and the PCM FCD was always scaled to be 15% larger than in the surrounding ECM. Overall, the simulated cell change in volume was more sensitive to the collagen orientation in comparison with the FCD (Figure 7.9). The cell volume change was clearly linearly related to middle zone thickness, in such a way that the reduced middle zone thickness led to an increased cell volume loss. Decreasing the superficial zone thickness had a similar effect, while increasing the thickness from a reference level
of 5% exerted only a negligible effect on the cell volume change due to compression.

**Figure 7.9:** Parametric analysis on how fixed charge density (PG content) and zone thicknesses (collagen orientation) affect cell volume change due to loading. Dashed line indicates implemented chondrocyte fixed charge density. Markers are model outputs and are connected by spline interpolation for illustrative purpose. Variation in models (min - max) versus variation in experimental data (mean ± 95% CI) is also presented. Model of the medial femur was used as the baseline model.
8 Discussion

The structure and composition of articular cartilage, i.e., the tissue’s response to indentation loading, and the subsequent in situ cell deformations, were investigated across various locations within the rabbit knee joint – in healthy animals and in both the contra-lateral and operated knee joints at 3 days following a partial meniscectomy in one knee joint in each operated rabbit.

8.1 CELL-TISSUE INTERACTIONS IN HEALTHY RABBIT KNEE JOINT CARTILAGE

The response of cells and cartilage was highly site-dependent on the delivered 2 MPa loading (Figure 7.1). Before going into details, it is worthwhile noting that we calculated nominal strains for the tissue to permit comparisons with previous works (such as [24,31,32]), even though the use of a different strain metric for large strains, such as Green-Lagrange strain, could be more appropriate as it eliminates rigid body rotations. By exploiting some parameter other than nominal strain, this would change the absolute strain values, but would not affect the conclusions. With this in mind, the changes in the cell height followed the magnitude of local nominal axial tissue strain, whereas the cell width and depth changes followed the magnitude of local nominal major and minor transverse tissue strain, respectively. In addition, the local nominal axial tissue strains were greater than the associated cell height changes for the delivered loading. This relationship between the cellular-level strain and the local ECM strain magnitude is consistent with previous observations at varying global strains from 10% to 50% [122]. This is something of a controversial finding, as the surrounding ECM is many times stiffer than the chondrocyte [287]. Moreover, in the study of Choi et al. (2007), when the global axial tissue strain was less than 30%, the cell height strains were higher than the local axial ECM strain in the middle and deep zone cartilage – which is in contrast to the situation in the superficial layer [122]. Hence, the apparent cell height deformation seems to be dependent on the magnitude of the axial tissue strain and the cartilage zone. It has been postulated that this behavior is due to the PCM and, at least partly, to its zonal uniformity in stiffness [112,118]. As the ECM stiffness increases with cartilage depth [121], the ECM to PCM stiffness ratio will concurrently change, and this could explain why the relatively stiff PCM dampens cell strains in the superficial layer while amplifying them in the deep layers of cartilage at low local strains (< 30%) [22,103,122]. However, at higher tissue strains, the PCM becomes deformed more than the chondrocyte, regardless of cartilage zone, possibly shielding the cell from excessive strain [122]. A similar observation has been made in a computational study of osteoarthritic cartilage, where it was observed that reducing the PCM stiffness caused the PCM to undergo higher deformations, while reducing the chondrocyte strain [280]. The current observations of cell strain and axial tissue strain support the proposed protective effect of the PCM in the superficial layer [112,122,180].

As far as is known, this is the first time when the depth-wise composition of the
cartilage ECM and the pericellular PG content have been compared between contacting knee joint regions, and the relationship of the aforementioned compositional parameters examined with the chondrocyte deformations. The main finding was that knee joint regions with smaller chondrocyte volume and dimension changes (i.e., femoral groove, lateral tibia, medial femur) had a higher local collagen content, a higher PG content, a lower collagen orientation angle or a combination of these factors. In contrast, the opposite was observed in the patellar, lateral femoral and medial tibial cartilages (Figure 7.1 and 7.2). These findings were also supported by the regression analysis. A high collagen content in the local matrix was predictive of a lower cell height reduction and cell width elongation during compression, and this occurred in such a proportion, that the change in the cell volume (volume loss) was also reduced. Similarly, a high PG content was associated with a reduced cell volume loss due to compression. In addition, a low collagen orientation angle (orientation parallel to cartilage surface) correlated to a lower cell elongation and axial compression due to tissue loading. Furthermore, the PG content was greater in the PCM as compared to the surrounding ECM at every anatomical location. Based on the current FE model investigations and previous theoretical observations, increasing the pericellular FCD (while keeping unaltered the ECM FCD) will result in increased cell volume changes [258]. Interestingly, the difference between the PCM FCD and the surrounding ECM FCD was observed to be greatest in the patellar and medial tibial cartilages. This factor could be responsible for a greater mismatch in the PCM to the ECM stiffness and contribute towards larger cell volume changes due to compression at these sites [93, 112, 288].

The observed role of collagen content and fibril orientation in the regression models is in agreement with previous studies that have revealed the importance of collagen network on the cartilage indentation response [289–292]. The higher collagen content in the ECM could decrease the local strains by making the ECM stiffer. This would restrict cell elongation and provide support against cell height reductions, whereas the opposite applies to scenarios where there are lower amounts of superficial collagen. A similar reasoning can be applied to collagen fibril orientation, when the fibrils are oriented in parallel to the surface (i.e., the orientation angle is low), there will be much more tension generated in the fibrils and the resistance to compressive tissue loads will be higher. If the orientation angle is high, the main axis of the collagen fibrils will be more parallel to the compressive forces. This will cause an increased local tissue strain and subsequent cell height and width changes, as collagen fibrils yield easily under compression along their axis [55, 238, 242, 293]. The association of a high PG content to lower cell volume changes is consistent with previous theoretical observations [258], and with the current FE model results (Figure 7.9). Since the PG content is associated with the equilibrium stiffness, it could be that cells in regions of high PG content will be less compressed in all directions.

The cause of site-dependent cartilage structure is likely a result of functional tissue adaptation to meet the different biomechanical requirements during early life, as articular cartilage is known to be biomechanically homogeneous at birth [294]. One single study of rabbit gait [295] reported that the lateral compartment of the rabbit knee joint was preferentially loaded, which could be one of the reasons for the observed high stiffness of the lateral tibial cartilages. This could also be the cause for the relatively thick superficial and middle zones in the lateral tibia (Table 6.3). Other differences between knee joint sites were also evident, for example, patellar
cartilage has less PGs, less collagen, and less parallel collagen fibril orientation in the SZ as compared to femoral groove cartilage. This is in agreement with previous studies, which show that the patella is softer and more permeable than the femoral groove [296,297]. This was also observed when optimizing FE model parameters for these two anatomical sites ($E_m$ and $k$ in Table 6.3).

### 8.2 EARLY EFFECTS OF PARTIAL MENISCECTOMY ON ARTICULAR CARTILAGE AND CHONDROCYTES

When comparing the PM group to the CNTRL or the C-L group, the chondrocyte volume changes due to indentation loading were decreased, increased or unchanged, depending on the knee joint location. Smaller cell volume changes in the PM group were observed in the medial side of the knee joint, in both femoral and tibial cartilages, whereas larger cell volume changes (increased loss of cell volume) were evident in the patellar, lateral femoral and femoral groove cartilages. Interestingly, chondrocytes from the lateral tibial cartilage showed similar volume changes between the groups. Sites with significant alterations to cell volume behavior had decreased the superficial PG content in the PM group compared to either the CNTRL or the C-L group, whereas the collagen content or collagen fibril orientation angle were not significantly altered in any site. In addition, the tissue strains or equilibrium moduli of cartilages were not significantly altered 3 days after the PM surgery.

The trend towards smaller cell volume changes due to the PM surgery, as observed in the medial femoral and tibial cartilages (Figure 7.5), is similar to that observed in rabbit patellar chondrocytes at 4 or 9 weeks after an anterior cruciate ligament transection (ACLT) [31, 32]. This is illustrated in Figure 8.1, which illustrates measured cell deformations from these previous publications [31, 32] together with a subset from the current results.

**Figure 8.1:** Cell morphology changes due to cartilage loading from the current and previous studies. In addition to the patellar cartilages (the studied location in [31, 32]), responses of chondrocytes from the medial tibial cartilage are shown, as the volume change was altered due to PM in a similar way as previously due to ACLT. Mean values and 95% confidence intervals are presented.
In the previous studies, the mean chondrocyte volume changes in contra-lateral groups were −5% and −8%, whereas the mean cell volume increases of 24% and 8% were reported in the 4-week and 9-week ACLT groups, respectively (Figure 8.1). In addition, a superficial PG loss was observed at both time points after ACLT, as was noted here, but this was already accompanied by observable alterations in the collagen network, hence representing a more advanced stage of articular cartilage damage [32,33]. However, in our patellar cartilages, we saw an opposite cell volume behavior compared to these two studies – the mean cell volume loss was increased from −12% to −20% due to the PM. The loss of PG content in the ECM is likely contributing towards this increased cell volume loss observed in the patella, femoral groove and lateral femur here. The link between the loss of the ECM PG content and the increased cell volume loss would be consistent with the current regression analysis (Table 7.1) and FE modeling results (Figure 7.9), in addition to previous experimental and numerical analyses [33,258].

Hence, the trend towards increased cell volume after loading in medial femoral and tibial cartilages observed here and previously in patellar cartilages is not likely caused by the loss of the ECM PG content. Other reasons for the increased cell volume (which we have not quantified and are purely hypothesized) could include alterations in the mechanical properties of the cell or the PCM, or to the cell’s capabilities to regulate volume. First, chondrocytes from osteoarthritic human cartilage have been reported to be stiffer than cells from visually healthy cartilage, possibly due to alterations in the cell cytoskeleton [287,298]. This could cause reduced cell height changes, as observed in both 4-week and 9-week ACLT studies (Figure 8.1). In the current study, this would be consistent with the behavior observed in the medial femoral cartilage, where the change in the mean cell height was observed to be −23% in the CNTRL group and −16% in the PM group. On the other hand, the stiffness of the PCM seems to decrease in osteoarthritic cartilage [93,203]. As the PCM can be considered to resemble a capsule surrounding the chondrocyte, its main function could be to resist lateral cell strains [299]. The loss of PCM stiffness would be consistent with increased lateral strains observed in previous ACLT studies [31,32] and computational simulations with loose PCM collagen fibrils [103]. This could be one pathway contributing to the observed behavior in medial tibial cartilage, where the change in the cell width was 12% in the CNTRL group compared to 17% in the PM group.

The increased lateral cell strains may be unfavorable, as excessive (≈ 20%) tensile strains of chondrocytes have been reported to induce matrix degrading enzymes and inflammatory factors [300,301]. On the other hand, the tensional strain of superficial chondrocytes is also linked to increased PG synthesis [302], but this was obtained with 1 Hz, 5% sinusoidal loading. Hence, it is likely that any alterations in cell deformations may affect the synthetic capabilities of the cell. The last mentioned pathway i.e., via altered volume regulation, is also possible, as normal chondrocytes exhibit a regulatory volume decrease [196,197,303]. During OA, the chondrocytes swell, and this swelling exceeds the amount that can be attributed to the increased cartilage water fraction (assuming that chondrocytes act as perfect osmometers [194]) suggesting that regulation of volume is in some way altered [153]. In addition to the volume regulation or changes to the properties of the cell or the PCM, it is also possible that the collagen network fibril-level organization, such as inter-connectivity or crosslinking, becomes altered, although we could not observe this with current methods [177,293]. Minute changes to the collagen network in the cell vicinity could alter cellular deformations without
significantly affecting bulk tissue mechanics.

In addition to the ECM PG content, we also examined the pericellular PG distributions (Figure 7.7). The absolute changes in the PCM PG content due to PM were rather similar to the ECM-level changes in each knee joint location. However, the mismatch increased between the PCM and the surrounding ECM PG content due to the PM surgery, which may affect the mechanical coupling of the cell-PCM-ECM complex. Similarly, a mismatch in the PCM to ECM PG content was evident at 9 weeks after ACLT [33], and it also has been reported to be present at 4 weeks after ACLT [304]. This could be due to increased PG synthesis by the chondrocytes in the damaged tissue, or due to the fact that PGs are being more strongly bound or are more resistant to degradation in the PCM compared to the ECM, or due to a combination of these possibilities. The higher FCD in the PCM compared to the EOM would lead to a higher cell volume loss, according to the current FE model (Figure 7.9) and to previous numerical studies [103, 258]. Thus, extensive loss of PCM FCD would actually cause an increase in cell volume, as seen here in the medial femoral and tibial cartilages.

Overall, these observations suggest that alterations in chondrocyte responses and the loss of superficial PG content after partial meniscectomy may precede macroscopic changes and significant alterations occurring in the biomechanical properties of the articular cartilage. The abnormal deformation of cells is associated with the proteoglycan loss and the down-regulation of synthesis [305–307]. Sufficient loading of chondrocytes is required for healthy cellular synthesis, whereas mechanical over-straining of chondrocytes involves a production of pro-inflammatory mediators, which may initiate tissue degeneration [308–310]. Hence, it is evident that any alterations to the normal habitual loading of the articular cartilage, and subsequent chondrocyte deformations, may lead to progressive damage of the articular cartilage. The current observation of superficial PG loss is consistent with the findings of Colombo et al. (1983), who also observed a PG loss in the rabbit knee joint 3 days after lateral meniscectomy [163]. As we did not have a sham-surgery group, it remains an open question if post-surgical inflammation could be the sole cause of the observed superficial PG loss. This, however, is not likely, as multiple previous studies have reported a lack of response in sham-operated knees [159, 168, 311], including the study of Colombo et al. [163]. Naturally, the presence of post-surgical inflammation can hasten tissue degradation and PG loss by down-regulating the production of aggrecan [10, 79].

### 8.3 Computational Simulations of Cell Deformation Experiments

The FE simulations were able to reproduce chondrocyte volume behavior with fair accuracy (Figure 7.8). Furthermore, the site-specific differences between the PM and the CNTRL group and the simulated cell volume changes had a similar trend as observed in the experiments, e.g., increased cell volume loss in the patellar cartilages and decreased cell volume loss in the medial tibial cartilages. Hence, the altered cell volume behavior due to PM is, at least partly, due to changes in the structure, composition, and loading of the tissue. The magnitude of cell volume changes is not precisely captured by the FE models, but this was not the reason why these models were utilized here. In addition, this could likely be achieved by optimizing the PCM and the chondrocyte properties, which were now taken from the literature,
and assumed to be constant between the groups and the sites.

The main rationale for using FE models was to determine if and how measured composition and structure differences affect the site-specific cell volumes after loading. Because of this, it was possible to incorporate some simplifications into the FE model. First, the geometry of the model is simplified, as only a single chondrocyte is modeled, and axisymmetry is assumed. The cell distribution can affect the load-response of individual cells to some degree [26,312], but negligible effects due surrounding cells have also been previously assumed to be due to the rather sparse distribution of cells in articular cartilage [288,313]. Axisymmetry is another simplification of the native cartilage, but as with the cell density, it would have been challenging to accurately describe the anisotropy in each location (and group), and how the cells are possibly oriented due to this anisotropy. There are also some uncertainties with respect to the properties of the ECM, PCM and the cell, as part of these were based on values taken from the literature. Hence, possible alterations between knee joint sites and groups are not captured. In addition, the material behavior could be more realistic, for example, by modeling collagen fibrils with non-linear properties, but this would have required a multi-step indentation protocol in order to obtain a unique set of material parameters [261].

Even with these limitations in mind, the current simulations clearly imply that the loss of PGs in the cartilage ECM would lead to a greater loss of cell volume, whereas the loss of PCM PG content could have an opposite effect on cell volume (Figure 7.9). Both of these effects are illustrated in Figure 8.2. Interestingly, a minimal cell volume loss occurred when the FCD in the PCM and cell were similar. In this condition, a minimal swelling pressure gradient is present between the cell and the PCM, which subsequently means less tension in the PCM fibrils. This could allow greater cell swelling after loading, at equilibrium. These observations of the PCM and the ECM FCD are consistent with previous simulations [103,258]. Overall, FCD alterations in any part of the cell-PCM-ECM complex are likely present in damaged articular cartilage and do affect chondrocyte deformations.

The effect of FCD alterations were, however, rather small compared to how alterations in collagen network orientation affected changes in cell volume (Figure 7.9). This is consistent with previous observations, i.e., indentation loading causes a high local stress and strain concentration in cartilage, which are mainly resisted by the collagen network [290–292]. The effect of orientation, however, might be exaggerated due to the axisymmetry in the model, as the collagen network is now equally stiff in all transverse directions, whereas in reality, there is anisotropy in parallel and perpendicular to the local split-lines [314]. There were also slight differences in the FE models between groups in collagen content, thickness and applied axial tissue strains, as these were taken into model as average values from each site and group. The lesser amounts of collagen content are related to amplified cell volume loss (Table 7.1). Secondly, as collagen orientation was modeled with superficial and middle zone percentages (Figure 6.2), the thickness variation between groups will result in a slight variation in the absolute superficial and middle zone thickness (Table 6.3). Finally, small variations in compressive strain were able to affect the cellular response. Quite interestingly, higher axial tissue strains seemed to produce lower cell volume losses due to greater cell elongation in the transverse plane. Altogether, the ECM and PCM FCD losses seem to compensate for each other with respect to their effect on the changes in cell volume. On the other hand, the slight differences in cartilage structure and
absolute compression between the CNTRL and PM groups are able to trigger the simulated cell volume response towards an agreement with the experimental observations.

In the future, it would be interesting to determine the extent to which the chondrocyte response will change if the model includes a realistic 3D distribution of cells. In addition, the cell has now been modeled as a passive material but in reality, cells can actively respond to forces by modifying their cytoskeleton [193]. Based on active modeling frameworks, it seems that an inclusion of the cell cytoskeleton would be important if strain and stress in the cell nucleus are to be modeled [259, 315]. In addition, it would be interesting to examine the extent to which the chondrocyte deformations would change if cells were to be included in adaptive FE models of the articular cartilage, such as presented in previous studies [316–318]. In the future, it would be also interesting to determine how the
properties of the PCM collagen network are altered during osteoarthritis. This could be potentially evaluated with high resolution vibrational spectroscopic methods such as Raman spectroscopy or attenuated total-reflection FTIR [211,319], or by using high resolution imaging techniques such as transmission and scanning electronic microscopy [55,320]. The results of this thesis could be used when assessing the quality of cell-based tissue engineered cartilage, by comparing the cell deformations in the engineered scaffold to the situation observed in cartilages in situ. The tissue engineering platform could also allow interesting studies to be conducted on how and if selective digestion of molecules, such as collagen type VI or perlecain that are found in the PCM, would alter the way that cells become deformed. The former possibility is particularly interesting as PCM destruction has been postulated to occur during osteoarthritis [321].
9 Summary and conclusions

In this thesis, the interrelationship between chondrocyte biomechanical responses and articular cartilage composition and structure was investigated in healthy rabbit knee joint cartilages and in the same tissues at 3 days after a lateral partial meniscectomy. Experimental measurements of chondrocyte deformation due to tissue loading were performed and articular cartilage composition was quantified by histology. These measurements were later simulated using a multi-scale finite element model.

The following conclusions can be drawn based on this work:

1. Chondrocyte deformations due to indentation loading are controlled by the local tissue strains, and by the composition and structure of the articular cartilage. This causes the cell deformations to be highly site-dependent within the knee joint under a constant loading protocol.

2. In terms of composition and structure, the site-dependency of chondrocyte deformations was more strongly related to the local collagen content in the extracellular matrix and orientation angle of the local collagen network as compared to the local proteoglycan content.

3. Articular cartilage proteoglycan content and chondrocyte deformations are already altered a mere 3 days after partial meniscectomy in the rabbit knee joint, but these alterations take place in a site-specific manner. In addition, this occurs without signs of micro-level collagen network damage.

4. According to the simulations, the loss of proteoglycans in the extracellular matrix could increase chondrocyte deformations, whereas the observed loss of the pericellular proteoglycan content would have an opposite effect.

Based on the current observations, it is evident that alterations in rabbit knee joint articular cartilage occur rapidly after a partial meniscectomy. The first alterations seem to consist of a loss of proteoglycans and altered cellular deformations. Based on the experimental results from different knee joint sites in healthy rabbits, and based on computational simulations, damage to the collagen network would be likely to cause even larger changes in the chondrocyte deformations than those observed here at 3 days after the PM. This is consistent with the concept that macroscopic collagen damage (fibrillation) may represent a point of no return with regard to the potential for cartilage repair. In the future, it would be important to assess if and how much the collagen network of the PCM differs between different sites and groups. This could potentially explain some of the site-dependency observed here. It should be noted that the current analysis was purely biomechanical and restricted to the equilibrium state under conditions of static loading. This raises one interesting question; by how much will the cell deformations be altered due to a partial meniscectomy when there is dynamic loading, which could be physiologically a more important parameter. Hence, the
next challenge that we could tackle is to develop methods that would allow simultaneous quantification of chondrocyte bio-synthetic responses and mechanical deformations.
BIBLIOGRAPHY


Chondrocytes respond to mechanical stimuli by regulating their synthesis, but fail to do so during osteoarthritis, leading to articular cartilage degeneration. In this thesis, we used a partial menisectomy rabbit model of knee osteoarthritis to study how cell biomechanics and articular cartilage structure are altered as early as 3 days after surgery. The findings provide valuable information, which can be useful for understanding, predicting and preventing osteoarthritis progression.