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Multiaxial Mechanics of Human Articular Cartilage in Health and Osteoarthritis

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Cartilage covers the articulating bones in all synovial joints throughout the body, providing a wear-resistant, low-friction surface. Complex interactions of solid (collagen and proteoglycans) and fluid (water and dissolved ions) maintain the tissue’s load-bearing properties. Osteoarthritis burdens approximately 20% of the population in the United States, and its prevalence is projected to increase. With progressing disease, the tissue’s constituents alter in composition and structure, resulting in a downward cascade of degeneration.

In this work we determined the location dependent multiaxial properties of human cartilage. We found strain dependent anisotropy and significant inter-joint variability in mechanical strength and energy dissipation in healthy human tissue. Subsequently we investigated the mechanical response of tissue harvested from patients undergoing total knee arthroplasty. We correlated mechanical metrics with tissue composition and structure, assessed biochemically and histologically. We found a significant reduction in mechanical strength with moderate disease and a reduction in anisotropy. More importantly, we observed a significant reduction in energy dissipation, preceding changes in tissue composition, in tissue representing the onset of the disease.

A detailed analysis of the through-thickness shear strain distribution, obtained via digital image correlation, revealed significant changes in the depth-dependent mechanical response. Tissue deformation in the bottom 30% of the thickness reduces with mild structural remodeling and disease progression. Those changes in the mechanobiological environment of the tissue’s cell might further deregulate the cell response and progress the disease.

We successfully measured depth-dependent multiaxial properties of cartilage undergoing large strains and identified two potential biomarkers for the early detection of osteoarthritis.
Multiaxial Mechanics of Human Articular Cartilage in Health and Osteoarthritis

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BSc, Graz University of Technology, 2011
MSc, Graz University of Technology, 2014

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Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy
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Multiaxial Mechanics of Human Articular Cartilage
in Health and Osteoarthritis

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1 Introduction on Articular Cartilage

The musculoskeletal system, consisting of active (muscles) and passive (skeletal system) components ensures the mobility of the human body (Özkaya and Nordin 1998). Joints form the connection between rigid bones, allowing them to move relative to each other. The three main types of joints, with increasing degree of mobility, are synarthrodial, amphiarthrodial, and diarthrodial joints (Speckmann and Wittkowski 2004). For example, synarthrodial, or fibrous joints form a relatively rigid connection and connect the cranial bones to form the skull. Amphiarthrodial, or cartilaginous joints allow for some relative movement and feature connective tissue, i.e. ligament or cartilage, to separate the bones. This type of joint connects, for example, the ribs to the sternum or forms the intervertebral disc. The last type, diarthrodial or synovial joints, are mechanically the most significant for joint articulation, allowing large relative movements. Joints like the knee, hip or shoulder fall in this category.

A ligamentous capsule encloses the space between the articulating bones, referred to as articular cavity, of a synovial joint. The synovial membrane lines the inner surfaces of the joint, providing the required nutrients to the avascular tissue within the joint by secreting synovial fluid. The synovial fluid distributes the nutrients throughout the joint and serves as a lubricant to reduce friction between the articulating surfaces. Articular cartilage lines those surfaces, providing a low friction and wear resistant coating. In some joints additional cartilaginous tissue separates the articular cartilage surfaces, either completely (discus) or partially (meniscus), serving as additional damping and aiding with load distribution.
The following thesis provides a detailed introduction on articular cartilage, focusing on the knee joint, in health and disease. This is followed by a description of the original research conducted by the author and concludes with an outlook towards potential adaptations and expansions of the experimental setup.

### 1.1 Composition

Articular cartilage is a multi-phase tissue consisting of fluid (water and dissolved ions) and solid (collagen, proteoglycans, other proteins and chondrocytes) constituents. Articular cartilage has neither blood nor lymphatic vessels, nor nerve fibers. It is separated from the underlying vascular subchondral bone by the tidemark, a dense layer of calcified cartilage.

Collagen fibers are the most common protein in the solid phase making up 10-20% of the wet weight. Collagen type II fibers represent 90-95% of the total collagen, forming the backbone of the extra-cellular matrix (ECM) (Sophia Fox et al. 2009). However, mature tissue further consists of types III, VI, IX, X, XI, XII and XIV and their interactions are essential to maintain the mechanical integrity of healthy tissue (Eyre 2002). Types IX and XI, with their high number of cross-linking sites, form a very stable heteropolymer with type II, contributing to the mechanical strength of the ECM. Collagen type III synthesis increases in response to matrix damage. Thus it might have a role in tissue remodeling and regeneration. The pericellular matrix surrounding chondrocytes, the only living cells within the tissue, show an increased collagen type VI content. Therefore this protein might play a role in cell-matrix integration. The function of the remaining proteins is not yet fully understood.

The other main component of the solid phase are proteoglycans (PG), accounting for 5-10% of the wet weight. Those large molecules consist of a core protein and attached glycosaminoglycane (GAG) side chains. The most common PG is aggregcan, which binds to hyaloronic acid as its core protein. There can be more than 100 side chains, forming a
1.1 Composition

Macro-molecule with a high fixed-charge density (Hardingham and Fosang 1992). These side arms give the macro-molecule its characteristic brush-like appearance in images obtained by electron microscopy (Buckwalter J. et al. 1985).

The fluid phase makes up 68-85% of the tissue’s wet weight (Mow et al. 2005). The dissolved ions, e.g. Na$^{2+}$, Ca$^{2+}$, and Cl$^{-}$ among others, within the fluid phase affect the osmotic pressure of the interstitial fluid and are considered a separate, electrically active phase of the tissue. The fixed negative charges of PG create a Donnan potential, pulling in ions dissolved in the interstitial water and increase the osmotic pressure (Wan et al. 2010).

Chondrocytes make up for approximately 4% of the tissue’s wet weight. This single cell type is responsible for synthesizing the different proteins of the ECM and thus regulating matrix formation, adaptation, repair and degradation (Akkiraju and Nohe 2015). They survive in a low oxygen environment and rely solely on diffusion to obtain nutrients from the synovial fluid. In healthy tissue new chondrocytes originate from differentiation from mesenchymal stem cells and populate cartilage throughout embryonic growth and development. In mature cartilage, stem cells originate from the bone marrow and enter the tissue through the articular margin at the periphery of the articular surface, a region that typically does not bear any load. There they multiply and differentiate into chondrocytes and migrate from the articular surface through the thickness of the tissue to the tidemark (Simkin 2008). At the tidemark, i.e. the front of reabsorption of calcified cartilage and ossification (formation of bone), the cells undergo apoptosis (Schultz et al. 2015). It should be mentioned that chondrocytes do not duplicate by mitoses in healthy tissue and are therefore not affected by telomere shortening, increasing the lifespan of the cells (Loeser 2009). Mechanical failure caused by chronic or high peak stresses seems to be the main trigger for cell senescence.
1.2 Structure & Distribution of Constituents

Constituents in cartilage show characteristic through-thickness distributions in structure and content. Figure 1.1 visualizes the alignment and shape of chondrocytes as well as the zonal ultrastructure of collagen fibers along a cross-sectional cut through the thickness of articular cartilage.

Collagen fibers form three distinct zones from the articular surface downwards described as the superficial (SZ), middle (MZ), and deep zone (DZ) (Clarke 1971, Mow et al. 1992). Each zone is characterized by a different collagen fiber composition, density, and alignment. The SZ, starting at the articular surface, spans over the top 10-20% of the total thickness. Thin fibers, with a homogeneous diameter around 50 nm (Changoor et al. 2011a), run predominantly parallel to the articular surface. Additionally, the fibers show an alignment with respect to each other depending on the location across the joint. This orientation is often visualized as the so-called split-line direction (SLD), the orientation of the principal axis of an elliptical hole formed by penetrating the articular surface with an ink-dipped circular pin (Below et al. 2002).

Underneath the SZ follows the MZ, which spans 40-60% of the total depth, and the DZ,
which makes up the remaining 30-40%. Fiber diameter increases with depth, with mean fiber diameters around 80 nm in the MZ and 110 nm in the DZ. Further, the fiber diameter distribution gets wider in MZ and DZ, with fibers ranging from 40-130 nm and 50-150 nm respectively (Changoor et al. 2011a). Collagen fibers have an isotropic distribution in the MZ and are perpendicular to the articular surface in the DZ. Little is known on the depth-wise collagen density distribution. Studies using animal models (Rieppo et al. 2009, van Turnhout et al. 2010) showed that the collagen content increases with depth, but no data exists on human patients. Data obtained from animal models can sometimes be generalized to provide insight in human tissue due to quantitative and qualitative similarities (Buckley et al. 2010). However, in this case it must be noted that the oldest animals investigated in the above studies were 72 weeks old. While animals reach skeletal maturity by that age, those investigations can not tell us about potential age related non-pathological tissue remodeling potentially affecting the collagen distribution occurring in humans simply because of the older age.

The large PG macro-molecules do not follow a preferred alignment or directionality, rather they are isotropic throughout the tissue. PG content is lowest in the superficial zone, increases in the middle zone and again slightly reduces in the DZ of the tissue. However, PG aggregates in the DZ appear larger and possess more aggrecan side chains than in the zones above (Mow and Guo 2002).

Water is distributed homogeneously over the tissue and has, by its nature, no directional alignment. However, the local water content is tied to the collagen and PG network. With higher PG content and thus a higher fixed charge density, the osmotic pressure is increased and water is pulled towards that region. It should be further noted that water cannot move freely through the tissue, but is restricted by collagen fibers. Thus the flow of water depends on the directionality of the surrounding fibers. Pierce et al. (2010) utilized this property to assess the in-vivo fiber distribution using diffusion tensor magnetic resonance imaging (DT-MRI).
Chondrocytes change in shape and density through the thickness. They appear flat and parallel to the articular surface in the SZ and become round in MZ and DZ. Further, they form column like structures in the DZ. Cell density is highest in the SZ and decreases towards the middle and deep zone (Gilmore and Palfrey 1988). With age, the total number on chondrocytes remains approximately constant. However, cells generally migrate towards deeper layers, reducing the amount of chondrocytes in the SZ and increasing numbers in the MZ and DZ (Hardingham and Bayliss 1990).

1.3 Mechanical Function

Interactions within cartilage among water, dissolved ions, a network of collagen, and densely packed, charged proteoglycans generate remarkable mechanical responses to loading. The ultrastructural arrangement and molecular interactions of the constituents of the extracellular matrix govern the deformation response of cartilage (Mow et al. 2005). The fixed negative charges of PG molecules within the matrix cause a repulsive force, pushing them apart. Additionally, an osmotic fluid flow pulls water molecules towards the PG molecules, causing the tissue to swell. The tensile stiffness of the collagen fibers and integrity of the matrix limits how much the tissue can actually expand (Mansour 2003). The resulting tissue swelling pre-stretches the collagen network, increasing the mechanical stiffness (Zhu et al. 1993).

External loads compress water through the articular surface out of the ECM (Linn and Sokoloff 1965), where low permeability (Maroudas et al. 1968) limits, and the osmotic pressure opposes, that flow. This mechanism makes cartilage act like an incompressible solid under fast \textit{in-vivo} loads, where water bears and transmits the load to the underlying bone while the actual solid matrix just restrains the water flow. Compression of the tissue causes high shear stresses near the bone-cartilage interface due to the difference in stiffness between cartilage and bone (Mansour 2003).
Zhu et al. (1993) showed that the shear stiffness of cartilage mainly originates from the dense network of type II collagen. Collagen fibers are relatively stiff under axial tension while they are easily bent or compressed (Li et al. 2005a, Yang et al. 2007, Buckley et al. 2008). Thus, collagen fibers under tension, aligned with the direction of deformation, primarily support applied shear deformations. Preferential alignment of the main load bearing component (i.e. collagen fibers), particularly within the SZ, suggests an anisotropic mechanical response to external loads. Wang et al. (2003) showed that the effective Young’s modulus and Poisson’s ratio varied when measured in different loading directions under compression. Robinson et al. (2016) also showed that the radial strain of the solid constituents under compression was anisotropic. These findings are consistent with uniaxial extension tests on cartilage strips harvested from the superficial zone where the mechanical response was significantly dependent on the fiber alignment in the SZ (Woo et al. 1979, Huang et al. 2005).

Interactions of the fluid and solid phase and between PG macro-molecules and collagen allow the tissue to dissipate energy (Zhu et al. 1993). These mechanisms play an important role for temperature regulation in the avascular tissue to provide an ideal thermal environment for chondrocytes (Abdel-Sayed et al. 2014b).

During normal movement cartilage generally experiences a combination of tension, compression, and shear. Physiological loading conditions cause large deformations within the tissue. In-vivo deformation measurements revealed compressive strains between 7-23% during the stance phase of gait (Bingham et al. 2008) or up to 30% during a weight-bearing single-leg lunge (Liu et al. 2010). Recently Chan et al. (2016) showed experimentally that shear strain generally exceeds compressive strain under compressive loading in vivo, highlighting the importance of a detailed understanding of the mechanical behavior under shear.

Loading conditions vary depending of the inter-joint location because of the wide range of motion of the knee joint and the presence of the meniscus, covering parts of the articulating
surfaces. There are load bearing regions, with cartilage surfaces of the femur and tibia in
direct contact and non-load bearing regions, covered by the meniscus (Blankevoort et al.

Mechanical loads applied to the bulk tissue alter the local mechanobiological environment,
stimulating a tissue remodeling response. This mechanotransduction affects synthesis and
degradation of matrix molecules (Grodzinsky et al. 2000). The cell response is sensitive
to the amplitude, frequency, and mode of the applied loading. Under homeostatic physio-
logical loading, chondrocytes maintained a balance between the degradation and synthesis
of ECM (Chen et al. 2013). Reduced mechanical loads reduce the activity of chondro-
cytes (Buckwalter et al. 2005), resulting in matrix loss and tissue erosion and can affect
the absorption-formation equilibrium at the bone-cartilage interface, resulting in tidemark
migration (O’Connor 2009). On the other side, supraphysiological loads can lead to lo-
cal tissue failure and cell death (Loeser 2009, Bartell et al. 2015). Within a physiological
range, increasing intermittent hydrostatic pressure upregulated PG synthesis (Smith et al.
2004) while cyclic tensile strains increased the amount of synthesized collagen (Wong et al.
2003). Shear deformations downregulated both PG and collagen synthesis (Smith et al.
2004). Further, Abdel-Sayed et al. (2014a) showed that in addition to mechanical loads
and deformations the amount of dissipated energy influences chondrogenic expression.
2 Osteoarthritis

Osteoarthritis (OA) is the most common type of arthritis and has afflicted 30.8 million patients (13.5% of the adult population) from 2008 to 2011 in the US (Cisternas et al. 2016). Projections estimate that by 2030, 25% of the adult population will suffer from this disease, where working-age adults will make up for one-third of the cases (Hootman and Helmick 2006). In addition to severe pain and a loss of mobility, OA patients show increased rates of insomnia, depression, and anxiety (Ferreira et al. 2015, Liu et al. 2018). Total patient cost accumulates to $303.5 billion a year, consisting of $139.8 billion in medical expenditures and $163.7 billion in earning losses (Murphy et al. 2018).

A meta analysis of 85 studies conducted by Blagojevic et al. (2010) identified an increased body-mass-index (BMI), previous knee injury, gender, age, intensive physical activity, certain occupational physical activities, and an increased bone mineral density as likely risk factors. Rossignol et al. (2005) investigated the effects of occupational exposure and found a five or six times higher incidence rate in women working in the clothing or cleaning industry, compared to the average population. This knowledge might help to prevent some instances of OA but the potential patient can not actively influence all of those factors. Thus, it is a necessity to obtain a more detailed understanding of the pathway leading to OA, to prevent initiation and develop treatments to stop and reverse its progression.
2.1 Pathogenesis

Post-traumatic (secondary) OA (PT-OA), i.e. disease initiation as a consequence of a joint trauma, accounts for approximately 12% of all symptomatic incidents of OA, however with great variations amongst different joints (Brown et al. 2006). The ankle joint is generally less likely to develop OA than the knee, with 1% vs. 15% of the population affected respectively (Valderrabano et al. 2008). Within the group of patients with symptoms in the ankle up to 78% developed those after traumatic injury, while only about 10% of OA incidents in the knee are post-traumatic. This also means that for roughly 90% of all patients with OA symptoms in the knee joint, no obvious incident triggering pathogenesis exists. Age related ‘wear and tear’ serves as a commonly used explanation for non-traumatic (primary) OA. But describing it as an abnormal, out-of-control remodeling process affecting all joint tissues, modulated by inflammatory mediators, might be more accurate (Loeser et al. 2012).

With progressing disease of the joint, articular cartilage and the meniscus erode, subchondral bone thickens, synovial inflammations spread, ligaments degenerate, and the joint capsule increases in volume. In addition to those tissues directly forming the joint, surrounding muscles, nerves, bursa, and fat pads can also degenerate and contribute to the degenerative cascade resulting in painful symptoms. In this thesis we focus on the degeneration of articular cartilage, one of the hallmark changes of OA and often associated with the onset of the disease. Degeneration and remodeling usually initiate at the articular surface in region with high \textit{in-vivo} loads and then progress deeper into the tissue (Hollander et al. 1995).

Despite the multifactorial nature of OA, mechanical stresses play a key role in the destructive evolution of the disease (Andriacchi et al. 2004, Loeser et al. 2012, Polur et al. 2010, Goldring and Marcu 2009). Both overloading (e.g. trauma) and reduced loading (e.g. immobilization) of cartilage induce molecular and microstructural changes that lead
to mechanical softening, fibrillation, and erosion.

### 2.2 ECM Degradation

Bobacz et al. (2004) reported that with OA the number of chondrocytes reduces by approximately 38% compared to healthy specimen, independent of age, resulting in a reduced protein synthesis for the tissue. However, cells harvested from OA tissue and cultured under homeostatic conditions did not show a diminished metabolism and synthesis rate. This suggests that changes in the chemical, biological, and mechanical environment, rather than pathological developments in the cell, alter the cellular expression.

Sandell and Aigner (2001) summarized the pattern of the chondrocyte cell responses in diseased cartilage in five general steps, starting with cell proliferation and apoptosis, followed by changes in the synthetic activity, degradation, phenotypic modulation and the formation of osteophytes.

Protein degrading enzymes up-regulated with OA and involved degenerative processes are metalloproteinases, serine proteinases, and cysteine proteinases (Troebberg and Nagase 2011).

Where the first seems to be the most significant one for matrix degradation with progressing disease, with A Disintegrin And Metalloproteinase with Thrombospondin motifs (ADAMTS) and Matrix Metalloproteinases (MMP) as most common and effective groups. ADAMTS-4 and ADAMTS-5 effectively cleave aggrecans and seem to play a key role in early disease, while MMP-3 increases in later stages (Song et al. 2007).

Fibrillar cartilage, i.e. collagen, resists enzymatic digestion well and only few enzymes can cleave it. Within cartilage MMP-13 is the primary collagenase (Billinghurst et al. 1997). Karsdal et al. (2008) reported that collagen degradation is irreversible while aggrecan degradation can be repaired, highlighting the importance of understanding and interfering with these destructive mechanisms to maintain an intact tissue. A variety of other met-
alloproteinas play a potential role in OA by activating other MMPs (Ra and Parks 2007) or by cleaving proteins where the fragments further increase catabolic activity (Zack Marc et al. 2009).

The majority of serine proteinases does not directly participate in ECM degradation but rather activate MMPs and ADAMTS. Only HtrA-1 (high temperature requirement A) degrades collagen type VI in the pericellular matrix (Polur et al. 2010), thus interfering with the cellular embedding, as well as aggrecan (Chamberland et al. 2009).

Among cysteine proteinases, cathepsin K, capable of cleaving collagen type I and II, plays an important role in degrading the cartilage ECM and subchondral bone (Jukka et al. 2005), but also regulates regular bone development (Fujita et al. 2000). Interestingly, this enzyme seems to also have a protective role for cartilage, as its down-regulation has been shown to upregulate MMP-13, thus increasing overall degeneration (Daisuke et al. 2009). Cathepsin B and D effectively cleave aggrecan and expression increases in OA cartilage (Fosang et al. 1992). Other enzymes from this group are calpains and caspases, with minor roles in OA degradation.

A variety of transcription factors effecting cellular pathways have been identified in OA joints (Drissi et al. 2005, Husa et al. 2010). One of them is runt-related transcription factor 2 (RUNX2), targeting MMP-13 and ADAMTS-4 and ADAMTS-5, which shows increased expression after mechanical injury (Tetsunaga et al. 2018). Cytokines, i.e. proteins important for cell signal transduction and associated with inflammation, also seem to be critical mediators regulating the pathophysiology of cartilage. Among this group, interleukin (IL)-1β and tumor necrosis factor (TNF) upregulate MMPs and ADAMTSs (Kapoor et al. 2010).

The synovial fluid might play a key role in how the disease spreads across the joint. It has been shown that it contains more matrix degrading proteins (Roach et al. 2005), thus transporting the enzymes to locations not yet affected by the disease. Further, its effectiveness as a lubricant is reduced, altering the boundary conditions and potentially increasing
mechanical loads at the articulating surfaces (Elsaid et al. 2005). The changes in chondrocyte expression, in combination with local traumatic overloads, result in progressing macroscopic ECM remodeling, affecting the tissues capability to withstand loads. This downward cascade of degradation accelerates and spreads tissue erosion and disease progression, causing severe pain and reduced mobility for the patient.

Macroscopic signs of ECM remodeling manifest as PG deaggregation accompanied by an increased osmotic pressure (Buckwalter et al. 2005) and collagen remodeling (Desrochers et al. 2012). Where the first reduces the tissue’s capability to withstand compression, and the latter, the tensile and shear shear stiffness. This further results in an increased water content and tissue swelling, and as a consequence, an increase in thickness (Adams and Brandt 1991). With progressing disease, PG depletes while the collagen content remains almost constant (Temple-Wong et al. 2009). However, collagen fibrillates and the zonal structure remodels resulting in a loss of alignment in the SZ and changes in the thickness of MZ and DZ (Changoor et al. 2011b). Remodeled or newly formed collagen has a reduced tensile stiffness and the overall structural integrity of the matrix diminishes progressively (Griffin et al. 2014). This is accompanied by an increase in surface roughness, culminating in the formation of fissures and tissue softening and subsequent tissue erosion (Buckwalter et al. 2005, Goldring and Goldring 2010).

The zone of calcified cartilage at the bone cartilage interface migrates upwards and multiple tidemarks can become evident with progressing disease (Mansfield and Peter Winlove 2012). Animal models of PT-OA, induced by anterior cruciate ligament transection (ACLT), have shown that the interface structure, i.e. roughness (Schultz et al. 2015) as well as the mechanical properties, i.e. elastic modulus and hardness (Pragnre et al. 2018), decrease. While this model represents traumatic overload, it has also been shown that the opposite, i.e. joint immobilization can accelerate tidemark duplication (O’Connor 2009). Finally, it can be seen that blood vessels start invading cartilage through the subchondral bone (Drissi et al. 2005), visible on histology slides as well as SHG images (Mansfield and
2.3 Diagnosis

Successful diagnosis of OA at early stages, prior to the development of clinical symptoms and serve damage to the connective tissues in the joint is of paramount importance to improve treatment effectiveness. Further, the development and verification of new treatment options needs tools to capture disease and regeneration accurately (Correa and Lietman 2017).

Evaluating histological sections allows for a precise detection of the through-thickness disease progression. A common combination of stains comprises Safranin-Orange (Saf-O) in combination with Fast-Green as a counter stain and Hematoxilin and Eosin (H&E). Saf-O stains PG while H&E stains cell cores, thus allowing a clear visualization of PG depletion and chondrocyte distribution. Toluidine blue yields similar results however, with less variation in color due to the lack of a counter stain (Schmitz et al. 2010). Picosirius red, a stain specific to collagen, amplifies the intrinsic birefringence of the aligned fibers. Observation of the stained slides under linear polarized light reveals the zonal architecture (Changoor et al. 2011b). Utilizing circular polarized light and thicker sections (approx. 50-100 µm) allows label free imaging of the collagen structure (Motavalli et al. 2014). Staging methods like OARSI (Pritzker et al. 2006) or Mankin score (Mankin et al. 1971) rely on those stains, where the first particularly resolves early stages of disease by design. The need for extracted, sectionable tissue limits the application of histological slides as a tool for in-vivo diagnosis.

Diagnostic arthroscopy is a minimally invasive surgical procedure allowing direct assessment of the articular surfaces. Typically, surgeons insert a camera by penetrating the joint capsule and scoring methods like Outerbridge (Outerbridge 1961) or SFA (Dougdos et al. 1994) provide established guidelines to visually quantify disease progression.
Those methods require microscopically visible fissures or lesions and they generally fail to detect early stages of OA. Only moderately reproducible and reliable results (Cameron et al. 2003, Menche 2003) emphasize the need for objective assessment tools. In a survey among surgeons, approximately 75% of the participants further expressed that need (Spahn et al. 2009). An alternative approach is to additionally insert an indentation tool to test the mechanical properties of the cartilage across the articular surface. Disease progression correlates with mechanical integrity but it again lacks sensitivity to detect the onset of OA (Brommer et al. 2006, Kiviranta et al. 2008).

Medical imaging techniques allow for non-invasive assessment of OA. With conventional radiography, cartilage appears almost transparent due to its poor absorption rate. Advanced image modalities, e.g. diffraction-enhanced imaging (Muehleman et al. 2009), can improve the visibility slightly. Most staging methods, as introduced by Kellgren and Lawrence (1957) or the International Knee Documentation Comitee (IKDC) (Irrgang et al. 2001) rely on joint space narrowing and remodeling of the subchondral bone as indirect measures to assess cartilage health. However, this does not allow observation of the articular surface roughness or structural integrity of the tissue and can underestimate loss of cartilage thickness (Amin et al. 2005), thus can not detect early stage OA.

MRI techniques can overcome those shortcomings, allowing direct measurements of cartilage morphology and composition (Guermazi et al. 2011, 2015). Semi-quantitative scores, e.g. Whole Organ Magnetic Resonance Imaging Score (WORMS) (Peterfy et al. 2004) or Boston Leeds Osteoarthritis Knee Score (BLOKS) (Hunter et al. 2008) allow reliable measurements of morphological changes (Roemer et al. 2009). However, minute changes in cartilage structure and composition, marking the very onset of OA, precede morphological changes.

Quantitative compositional MRI measurements, correlating with subtle signs of remodeling, provide promising results. MRI relaxometry uses T1ρ and T2 relaxation times, where the first is sensitive to PG depletion and the latter to water content and collagen fiber orien-
Osteoarthritis (Mosher et al. 2001, Duvvuri et al. 2006). Both values increase with degeneration, however vary naturally across the joint and through the thickness of the tissue (Burstein et al. 2009), which limits their capability as a diagnostic tool for local degeneration. However, over longitudinal studies, i.e. monitoring the same patient over a long period of time, both measures provide excellent resolution and sensitivity in detecting local changes.

Utilizing gadolinium as a contrast agent allows to image the intratissue GAG distribution and concentration with the delayed gadolinium-enhanced MRI of cartilage (dGEMRIC) method (Bashir et al. 1996). Intravenous injection of the contrast agent allows for an in-vivo application (Burstein et al. 2001).

Similar results can be obtained with GAG chemical exchange saturation transfer imaging (gagCEST), where the transfer of exchangeable protons is traced (Ling et al. 2008).

Diffusion tensor MRI makes use of the dependence of the intra-tissue water flow on the surrounding collagen network (Mlynárik et al. 2003). This allows to assess the intactness of the collagen architecture and correlates significantly with disease (Raya et al. 2013). Despite the tremendous potential, no metric so far has shown sufficient sensitivity to reliably detect the onset of OA.

Griebel et al. (2013) measured the through-thickness deformation patterns, rather than tissue composition, using the displacements under applied loading MRI (dualMRI) method on tissue explants. They found shear strains to increase significantly towards the center of the specimen with increasing degeneration. Higher sensitivity to degeneration than other metrics highlights the potential of this method (Griebel et al. 2014) however, the challenge to image cartilage in-vivo with a clinical MRI still remains.

Biomarkers in the serum or urine of OA patients, related to the initiation or progression of OA, might serve as a cheap, non-invasive alternative for disease diagnosis. All byproducts of cartilage degeneration, e.g. those described in Section 2.2 among others, serve as potential biomarkers. However, it remains challenging to gauge the stage of degeneration and to locate the origin of a partially metabolized biomarker to pin down the affected joint.
Currently biomarkers cannot aid towards the diagnosis or prognosis in individuals due to the lack of sensitivity, specificity and consistency (Lotz et al. 2013).

### 2.4 Treatments

A wide variety of potential treatment options for patients with OA symptoms exist and their applicability depends on the current stage of the disease. However, currently no treatment exists to fully restore cartilage (Correa and Lietman 2017). Early detection of pathological developments allows for a variety of non- or minimally-invasive treatments and improves therapeutic success rates. Orthopedic manual therapy in combination with exercise therapy, where the first focuses on joint mobility and range of motion and the latter on muscle strength and flexibility, provides short-term pain relief and an increase in joint performance (Abbott et al. 2013, Anwer et al. 2018).

Steroidal or non-steroidal anti-inflammatory (NSAIDS) provide short term pain relief but cannot modify disease progression (Fajardo and Di Cesare 2005). Newly developed drugs target degrading enzymes and cell signal transmitters (De Savi et al. 2011, Rudolphi et al. 2018, Steven et al. 2018) and provide promising results but have not yet reached clinical application.

Intra-articular injections of hyaluronic acid (HA) in combination with anti-inflammatory drugs can relieve pain (Euppayo et al. 2017). Exogenous HA can further increase the survival rate of chondrocytes, and endogenous HA and PG synthesis, and alters the ECM favorably, slowing down degeneration and improving regeneration (Moreland 2003, Altman et al. 2015).

Minimally-invasive surgical procedures to manage OA include arthroscopic lavage and debridement. Removing fragments of articular cartilage or crystals accumulated in the capsule by lavage reduces the inflammatory response Cameron-Donaldson et al. (2004).
Debridement describes resecting unstable cartilage and removing torn menisci and osteophytes (Felson 2010). The popularity of these procedures dropped dramatically after Moseley et al. (2002) found no efficacy of those treatments versus sham surgery (Lazic et al. 2014).

Full thickness cartilage defects and lesions can be treated by subchondral drilling or microfracture (Hamanishi et al. 2013). Damaging the bone-cartilage interface establishes an artificial blood supply that transports mesenchymal stromal cells from the bone marrow to the defect site. However, this procedure often leads to unwanted outcomes, e.g. formation of fibrocartilage or bone within the defect rather than hyalin cartilage (Frisbie et al. 2003), and can be considered a temporary solution at best (Gobbi et al. 2005).

Tissue defects up to 3 cm² can be treated with osteochondral autografts, i.e. the transplantation of cartilage from a healthy location in the joint to a degenerated one (Patil and Tapasvi 2015). The obvious drawback of this method is that the defect moves, rather than being regenerated, making follow up procedures almost inevitable. This method is mainly applicable for young, high demand patients, e.g. athletes having to return to their sport fast.

Advances in the development of biomaterials and a better understanding of the structure-function relationship prepare the way for tissue engineered cartilage replacements. A typical setup for engineered tissue consists of a cell type to synthesize new matrix material and a scaffold, serving as a template for growth and initial mechanical support. Either chondrocytes or mesenchymal stem cells serve as potential choices for cells. The greatest challenge for the use of chondrocytes poses harvesting and cultivating sufficient amounts, and for stem cells, the precise control of growth and differentiation. Hydrogels with tunable network density and composition have shown great promise to serve as a flexible scaffold, mimicking the solid and fluid phase of cartilage (Brandl et al. 2007, Zhang et al. 2009, Armiento et al. 2018).

Once cartilage erodes beyond repair only knee arthroplasty can relieve pain and restore joint mobility. A prosthetic made of metal alloys with low-friction, wear resistant artic-
2.4 Treatments

ulating surfaces, coated with polyethylene or ceramics, replaces the knee completely or partially. Unicompartmental knee arthroplasty shows a faster recovery rate (Price et al. 2001) and better cost-efficiency, especially for patients aged 65 years or older (Ghomrawi et al. 2015), than total replacement. However, this comes at the cost of a higher revision rate, mainly caused by implant loosening and pain (Kendrick et al. 2015). Typically, surgeons use bone cement to fix implants. This procedure provides an excellent fixation but comes with increased surgical time, potential thermal necrosis during cement hardening and technically demanding revisions of failed implants. New advancements with biomaterials and surface design improve osteoconductivity and allow for cementless procedures. However, they do currently carry an increased risk of loosening (Matassi et al. 2013).

Constant improvements in post-surgery treatment, e.g. deep tissue temperature regulation (Ueyama et al. 2018), can further improve the outcome of these procedures, making them a drastic, but successful final resort.
3 Mechanical Characterization: Literature Review

3.1 Compression Testing

Earliest descriptions of articular cartilage go back multiple centuries (Hunter 1743) but a detailed description of the tissue as a multi-phase material with depth-dependent properties started in the 1960s (McCutchen 1962, Maroudas and Bullough 1968).

Early indentation tests characterized the properties of healthy cartilage under static loading (Elmore et al. 1963) or mapped the time-dependent response of diseased joints by creep testing (Kempson et al. 1970). Those early tests showed significant softening in regions visibly affected by degeneration, correlating with a loss of PG content and associated with the development and progression of OA (Byers et al. 1970). Indentation tests allow measurements of the mechanical properties with in-situ boundary conditions, thus providing insight into the mechanical response of the intact joint. Advances in measurement techniques and refinements in disease staging led to the development of hand-held indentation tests used as diagnostic tools (Brommer et al. 2006, Kiviranta et al. 2008). Both studies confirmed disease induced bulk tissue softening, however not apparent at the initial phase of pathological tissue remodeling. Nano-indentation tests, utilizing atomic force microscopy (AFM) allows investigation of the constituents individually. Therefore a spherical, cylindrical or conical tip, attached at the end of a small cantilever beam, scans the
tissue and the measured beam deflection corresponds to stiffness of the individual collagen and PG molecules. Changes on the nano-scale precede apparent signs of remodeling and degeneration of the tissue’s morphology (Stolz et al. 2010). However, a detailed analysis of the mechanical data remains challenging since cartilage does not necessarily fulfill all the underlying assumptions for the required contact models, e.g. the Hertz model (Hertz 1882), for all test configuration.

Compression tests on cartilage explants either restrict expansion perpendicular to the loading direction, i.e. confined compression, or allow unconfined deformation. Confined compression testing enables measurements of the tissues permeability and the stress-relaxation properties, where the latter strongly correlates with the fixed charge density (Armstrong and Mow 1982, Mow et al. 1998, Torzilli et al. 1999). An applied external force pushes water through a porous filter out of the tissue, which sits in a tightly fitting well. The incompressible fluid phase dominates the initial mechanical response while the time-dependent response depends on the tissue’s capability to restrict the outward fluid flow. Higher permeability favors the outward water flow and a higher fixed charge density (PG density) increases the force required to push the water out. Once the setup reaches equilibrium under a constant force, the steady-state mechanical response of the solid matrix can be determined. With advancing degeneration the tissue’s permeability increases while the static compressive modulus reduces (Boschetti and Peretti 2008).

*In-vivo* boundary conditions allow for lateral expansion and while confined compression tests provide remarkable insight into the solid-fluid interactions, unconfined compression tests better represent the *in-situ* boundary conditions (Park et al. 2004). Unconstrained extension of the collagen fibers in the SZ further allows to investigate the intrinsic anisotropy (Démarteau et al. 2006) and its development with OA (Robinson et al. 2016). Tests performed at physiological loading rates, i.e. representing walking or running, reveals tissue fatigue and compaction (Kaplan et al. 2016). Instantaneous compression, i.e. impact, can simulate traumatic overloads and provide insight into pathological developments like local
tissue failure or chondrocyte death (Atkinson et al. 1998b, Kaleem et al. 2017).

3.2 Tension Testing

Studies performing tension tests typically focus on the mechanical response of the individual cartilage layers. Dumbbell-shaped specimen help to create a region of homogeneous stress-distribution in the center of the specimen and reduce the stress concentration near the clamps, due to the larger contact surface. Experiments extending cartilage strips show a nonlinear stress-strain response, with depth-dependent stiffness and anisotropy. The stiffness reduces depth, i.e. from the SZ towards the DZ. Specimens harvested from the SZ and MZ respond stiffer when stretched in SLD than perpendicular to it, while specimen from the DZ are isotropic (Woo et al. 1976, 1979). Biochemical analysis showed that the mechanical response of a specimen extended in SLD correlated with the collagen content, while it does not for specimen tested perpendicular to the SLD. This effect vanished with increasing depth. Further, PG content does not correlate with tensile properties (Kempson et al. 1973), highlighting the individual contribution of the constituent towards the bulk material properties.

Kempson (1991) found that the change in fracture stress and tensile stiffness with age varies between joints. Both mechanical metrics for the SZ and MZ significantly decrease with age in tissue from the femoral head. Fracture stress of cartilage from the ankle does not change and tensile stiffness only slightly reduced for the SZ and even increases for the MZ. This could offer a potential explanation why the ankle generally has a low OA incidence (Valderrabano et al. 2008).

Huang et al. (2005) confirmed this depth-dependent, anisotropic response and further reported a significant tension-compression nonlinearity. Tensile modulus was two orders of magnitude larger than the aggregate compressive modulus, i.e. the compressive stiffness of the solid constituents, across all zones. This emphasized the importance of the fluid phase
for bearing compressive loads while the solid constituents have a supporting and confining role under this loading mode.

On a smaller scale, devices like AFM and optical tweezers can test the mechanical response of individual collagen fibers or molecules. Bhole et al. (2009) adhered collagen type I fibers to the tip of an AFM and found that the protein withstands enzymatic digestion better when under tension. Utilizing optical tweezers, where optical beads adhered to the ends of a molecule can be moved by a focused laser, Sun et al. (2004) stretched individual collagen type II molecules. They found sharp non-linear stiffening once extension reached the molecule’s contour length.

3.3 Shear Testing

Comparably few studies investigated articular cartilage under shear. Cartilage undergoes a combination of different loading modes in-vivo. The complex zonal architecture of cartilage results in a heterogeneous response within the tissue to externally applied loads. For example, bulk compression sets fibers in the SZ under tension and the difference in stiffness between the DZ and the underlying bone introduced high shear stresses at the interface (Mansour 2003). Further, excessive shear loads modulate chondrocyte metabolism (Smith et al. 2004), can trigger apoptosis, and facilitate the development and progression of fissures in the ECM (Atkinson et al. 1998a).

Hayes and Mockros (1971) were the first to perform creep-compliance tests on articular cartilage under infinitesimal torsional shear. They reported an initial linear viscoelastic response and disease induced softening. Testing in torsion does not generate a fluid pressure gradient due to its isochoric nature, and thus provides direct insight into the intrinsic viscoelastic properties (Zhu et al. 1993).

In a subsequent study, Hayes and Bodine (1978) tested the dynamic response of enzymatically treated cartilage from 20-1000 Hz under infinitesimal torsional shear. Dynamic
measures allow for an evaluation of the energy stored and dissipated during deformation, i.e. calculation of the storage and loss modulus, respectively. They found that collagen depletion reduced both, storage and loss modulus, while PG depletion only affected the storage modulus. This shed light on the different roles of two main constituents in cartilage.

Simon et al. (1990) demonstrated irreversible fatigue under 3-5% sinusoidal simple shear at 100 Hz after approx. 180,000 cycles. They found a reduction in stiffness and dissipated energy and associated it with progressive damage to the collagen network and loss of PG. Tests at 0.01-20 Hz, i.e. physiologically motivated, with varying levels of precompression conducted on enzymatically treated specimen further increased the understanding of the structure-function relationships within articular cartilage (Zhu et al. 1993) under small strains. Collagen fibers under tension provide the shear stiffness of cartilage. The interwoven PG network increases the osmotic pressure, prestretching the collagen network and increasing its stiffness. They further established a combination of mechanisms responsible for energy dissipation within the tissue, i.e. interactions among the solid constituents (PG-PG and PG-collagen), as well as solid-fluid interactions (PG-water and Collagen-water), and internal viscous dissipation within the fluid.

A study designed by Nguyen et al. (2010) investigated cartilage under large applied shear strains. However, they investigated this in the context of the frictional properties where displacement of the articular surface was restricted only by the coefficient of friction of the opposing surface and otherwise free to slide. This results in an unknown applied displacement, thus they cannot guarantee constant testing conditions for different specimen, and as a result, cannot provide information about the stress-strain relationship. This data would be of great importance to improve and validate current mechanical models. They found that increased surface roughness in combination with increased precompression transmits higher deformations to the tissue. In a subsequent study they further found that cartilage stiffens with increasing applied precompression and an increase in osmotic pressure
The mechanical response measured by bulk tissue testing only represents averaged properties over the tested volume. The heterogeneous structure and constitution of cartilage causes strong through-thickness variations in the mechanical properties and hence responses. Digital image correlation (DIC) can resolve the spatial variations in displacement fields and can enhance experimental setups characterizing the mechanical properties of cartilage. Equipping bulk compression or shear tests with this non-contact, image based method can help to elucidate the bio-mechanical function of certain zones in the tissue. Therefore a camera setup records the deformation of a surface, where two or more cameras allow a 3D reconstruction. To obtain a full 2D displacement field the surface must have a stochastic pattern, either intrinsic or applied, which deforms with the material without affecting the material properties. For biological tissues the application of small particles, e.g. graphite powder or printer toner, or dyes airbrushed on the specimen, generally provide a sufficient pattern (Palanca et al. 2016, Zhou et al. 2016). Alternative approaches track the deformation of intrinsic features or applied lines on the cross-section to measure shear strains (Buckley et al. 2010).

Schinagl et al. (1997) determined the depth-dependent equilibrium confined compression modulus of bovine by tracking fluorescent cells. They found that the modulus progressively increases up to 15-fold from the superficial layers to the deeper zones. An optimized method for image analysis allowed Wang et al. (2002) to determine the depth-dependent apparent Poisson’s ratio in addition to the elastic modulus. They found that both follow the same trend, i.e. progressively increase from the articular surface to the bone-carriage interface. At the articular surface the Poisson’s ratio reached a lower limit of 0.01, attesting to the material’s remarkable tension-compression ratio at equilibrium in this zone. This

(Nguyen and Levenston 2012).
depth-dependence develops with tissue maturation, as immature tissue shows a homoge-
neous modulus. Subsequently, the compression modulus of the DZ increases ten-fold with
maturation (Gannon et al. 2015). A full-field analysis of the 2D strain field allowed for
measurement of the compressive, radial, and shear strain distributions under unconfined
compression (Lai and Levenston 2010b). Compressive strains progressively decreased
from the SZ inwards while radial and shear strains showed a distinct peak 100-200 µm
below the articular surface.

Similar to bulk tissue testing, the majority of experiments incorporating DIC apply com-
pression, while only few groups investigate cartilage under shear. However, shear deforma-
tion greatly reduces the out-of-plane deformation compared to compression, a significant
advantage when performing DIC. Buckley et al. (2008) measured the through-thickness
shear strain distribution of neonatal bovine tissue under small simple shear. They tracked
fluorescently stained cells to obtain the depth-dependent displacement, shear strain, and
shear modulus. Shear strain and modulus showed a distinct peak approx. 100 µm below
the articular surface, in the transition zone between SZ and MZ. Applied axial strain further
increased the prominence of this peak, indicating that axial strains weaken this transient
zone. Equilibrating the specimen after each increment of applied displacement did not al-
low dynamic measurements. In a follow-up study Buckley et al. (2010) tracked a set of
parallel lines, photobleached on the cross-section of the tissue, and found the same char-
acteristic peak in the transition zone of neonatal bovine and adult human tissue. In a series
of subsequent studies, this research group expanded their experimental setup to measure
the response under dynamic loading (Buckley et al. 2013), on specimen originating from
different locations across the joint (Silverberg et al. 2013), and on enzymatically treated
tissue Griffin et al. (2014). They further established a rigidity perlocation model, incor-
porating the depth-dependent collagen fiber orientation and density Silverberg et al. (2014).
The results of their experiments suggest a protective role for the transition zone between
SZ and MZ.
With a similar experimental setup Motavalli et al. (2014) demonstrated the dependence of the through-thickness shear strain pattern on the integrity and development of the collagen matrix. They found that with tissue maturation the location of highest shear strains transitions from the SZ deeper into the tissue. While their experimental results match previously reported findings for young, structurally immature bovine tissue this is not the case for mature tissue (c.f. Buckley et al. (2010)). This discrepancy could originate from differences in the loading protocol, foremost the application of large deformation.

By tracking a set of cell nuclei, Wong et al. (2008a) obtained the full-field deformation and strain field of healthy and diseased human cartilage under simple shear. They found shear strains to be highest at the articular surface and a three to five fold increase with degeneration. However, they did not report a distinct peak below the transition from SZ to MZ. With their setup they further investigated the influence of the lubricant and found that synovial fluid reduced the transmitted shear deformations (Wong et al. 2008b). They applied displacements by sliding a counter surface across the specimen, which does not allow to determine the exact amount of displacement applied to the tissue.

### 3.5 Open Questions and Research Motivation

Within the scope of this thesis we aim to expand the field of cartilage tissue mechanics. Studies showed that peak deformation can reach up to 30% compressive strain during regular exercises (Bingham et al. 2008) and that shear strains generally exceed compressive strains, even when bulk compression is applied (Chan et al. 2016). Hence mechanical testing at small strains (less than $3 - 5\%$) does not mimic the full *in-vivo* mechanical environment and thus fails to provide full insight into the mechanical responses of cartilage. Further, cartilage shows a strong directional structure in the superficial zone, the SLD. Preferential alignment of the main load bearing component (i.e. collagen fibers) particularly within the SZ, suggests an anisotropic mechanical response to external loads,
confirmed by uniaxial extension tests on cartilage strips (Woo et al. 1979) and unconfined compression tests (Robinson et al. 2016).

The combination of the general scarcity of large strain shear tests, in particular, the absence of cyclic testing with controlled boundary conditions, and the absence of information about the anisotropy under shear loads largely motivated the first aim of our studies. To close this gap, we determined the large-strain shear response of healthy human articular cartilage under multiaxial, cyclic deformation.

Large strain compression tests on OA tissue showed softening (Robinson et al. 2016) and an alteration of the viscous properties (Desrochers et al. 2012). Surface roughness increases (Pritzker et al. 2006) and the lubricating ability of the synovial fluid decreases (Elsaid et al. 2005) with disease. This increases shearing of cartilage during joint articulation, further increasing the contribution of this loading mode to the total deformation. However, no data currently exists for diseased cartilage under shear, motivating our second aim. Our baseline study on healthy tissue enables us to compare multiple mechanical parameters obtained from multiaxial experiments over the course of disease progression. Especially, the progression of energy dissipation with early OA has not been subject of investigation.

The heterogeneous through-thickness shear strain distribution depends on an intact collagen structure (Motavalli et al. 2014). It is well established that the tissue's architecture and composition undergo significant changes with progressing disease (Mow et al. 2005, Changoor et al. 2011b) and recent studies suggest that structural remodeling precedes other pathological events in the development of OA (Desrochers et al. 2012). Griebel et al. (2014) found that shear-strains, induced by applied compression, increase towards the center of the specimen in diseased tissue. However, the through-thickness shear strain distribution has not been measured under large applied shear deformations, with a focus on resolving the earliest stages of OA. For our third aim, we test the hypothesis that the shear strain distribution significantly alters with progressing disease and that those changes are sensitive to very early, preclinical stages of disease.
3.5 Open Questions and Research Motivation

We present the outcome of our experiments in the following chapters. Each chapter represents a stand-alone journal paper article that has either been published (c.f. Maier et al. 2017) or is submitted for publication.
4 Shear Deformations of Human Articular Cartilage: Certain Mechanical Anisotropies Apparent at Large but Not Small Shear Strains

4.1 Introduction

Articular cartilage is a multi-phase tissue consisting of fluid (water and dissolved ions) and solid (collagen, proteoglycans, other proteins and chondrocytes) constituents. The interactions among water, dissolved ions, a network of collagen and densely packed proteoglycans generate the remarkable mechanical responses of cartilage to loading (Mow et al. 2005). Through the thickness of cartilage the principal alignment of collagen within its network is often described by characteristic zones: superficial (SZ), middle (MZ) and deep (DZ) (Jeffery et al. 1991). With respect to the articular surface, collagen fibers are preferentially orientated parallel in the SZ, isotropically random in the MZ and perpendicular in the DZ (Clarke 1971, Mow et al. 1992, Lei and Szeri 2006). In the SZ this orientation is often visualized as the so called split-line direction (SLD), the orientation of the principal axis of an elliptical hole formed by penetrating the articular surface with an ink-dipped circular pin (Below et al. 2002).
Using a dual fluoroscopic imaging system to measure cartilage deformation in vivo Liu et al. (2010) reported that peak deformation varied between 7 and 23% during the stance phase of gait and Bingham et al. (2008) reported that peak deformation reached up to 30% during a weight-bearing, single-leg lunge. Hence mechanical testing at small strains (less than $3 - 5\%$) does not mimic the full in vivo mechanical environment and thus fails to provide full insight into the mechanical responses of cartilage. Large compressive strains also cause large shear stresses within cartilage, particularly near the bone cartilage interface (Mansour 2003). Recently Chan et al. (2016) showed experimentally that shear strain generally exceeds compressive strain under compressive loading in vivo, highlighting the importance of a detailed understanding of the mechanical behavior under shear. Additionally, shear deformations play an important role in the mechano-biological environment, impacting local tissue failure and both chondrocyte metabolism (Smith et al. 2004) and death (Atkinson et al. 1998b). Severe stress-strain environments within cartilage, especially at larger strain magnitudes, lead to the development and progression of degeneration, e.g. osteoarthritis (OA) (Guilak et al. 2004, Martin et al. 2004, Hashimoto et al. 2009).

Surprisingly little data exists on shear testing of cartilage at large strains. Uniaxial extension tests at large strains are published for both human (e.g. Kempson (1991), Elliott et al. (2002)) and bovine tissues (e.g. Woo et al. (1976), Roth and Mow (1980)), as well as confined and unconfined compression tests at large strains (e.g. Schinagl et al. (1997), Robinson et al. (2016)). Regarding shear, Wong et al. (2008a) and Nguyen et al. (2010) reported studies on human cartilage under large deformations, but both investigated this in the context of the frictional properties where displacement of the articular surface was restricted only by the coefficient of friction of the opposing surface and was otherwise free to slide. The applied shear in these studies was thus difficult to control and the test protocol may affect the measured bulk shear properties. Only Motavalli et al. (2013, 2014) performed simple large shear experiments (up to 25%) on bovine patellofemoral groove tissue using a rigid interface to the testing device. These studies focused on measuring the
spatial distribution of strain after full relaxation, not the stress-strain response under cyclic loading, and thus they do not provide information about viscoelasticity.

The time-dependent response of cartilage originates from (i) the fluid-solid (poro-elastic) interaction of the matrix with the interstitial fluid (Nguyen and Levenston 2012, Buckley et al. 2013), and (ii) the intrinsic viscoelastic response of both the collagen and proteoglycan molecules as well as their interactions (Hayes and Mockros 1971, Zhu et al. 1993).

While testing in torsion does not generate a fluid pressure gradient, and thus provides direct insight into the intrinsic viscoelastic properties, simple shear testing requires special considerations. Simple shear tests at quasi-static displacement rates, slow enough that no fluid pressure gradient forms, would ensure flow-independent results.

The ultrastructural arrangement and molecular interactions of the constituents of the extracellular matrix govern the deformation response of cartilage (Mow et al. 2005). Zhu et al. (1993) showed that the shear stiffness of cartilage mainly originates from the dense network of type II collagen. Additionally, collagen fibers are relatively stiff under axial tension while they are easily bent or compressed (Li et al. 2005b, Yang et al. 2007, Buckley et al. 2008). Thus, collagen fibers under tension, aligned with the direction of deformation primarily support applied shear deformations. Preferential alignment of the main load bearing component (i.e. collagen fibers) particularly within the SZ, suggests an anisotropic mechanical response to external loads.

Experimentally, Guilak et al. (1995) showed that chondrocyte deformation in the SZ depends on the SLD. Wang et al. (2003) showed that Young’s modulus and Poisson’s ratio varied when measured in different loading directions under compression. Robinson et al. (2016) also showed that the radial strain of the solid constituents under compression was anisotropic. These findings are consistent with uniaxial extension tests on cartilage strips harvested from the superficial zone were the mechanical response was significantly dependent on the fiber alignment in the SZ (Woo et al. 1979, Huang et al. 2005). However, no study has yet investigated potential anisotropy in cartilage’s bulk shear properties, and
mention of the SLD is generally neglected in mechanical tests. Silverberg et al. (2013) did report the local SLD, but then intentionally harvested specimens from isotropic regions to avoid anisotropy by design.

The mechanical responses likely vary under shear loading depending on location within the joint since in vivo loading varies along the joint surface (Song et al. 2014, Blankevoort et al. 1991) and the tissue adapts to external loading. Both Franz et al. (2001) and Garcia-Seco et al. (2005) reported such heterogeneity using compression testing of articular cartilage, and Abraham et al. (2011) reported similar results for shear testing of meniscus. Only Silverberg et al. (2013) reported on shear tests from load bearing and non-load bearing regions of articular cartilage, but this was done under small shear strains.

In this study we investigate the intrinsic shear response of human articular cartilage under a range of applied shear strains from 5-25%, and dependence of this response on both local split-line direction (anisotropy) and location within the joint (heterogeneity). In light of the preferential alignment of the collagen network, particularly within the SZ, we hypothesize that the shear response of cartilage is both strongly anisotropic and location dependent. Toward this hypothesis we test location-matched human cartilage specimens from adult human donors under multi-axial, large shear strains and perform statistical analyses of our results.

4.2 Materials and Methods

Using a triaxial shear testing device (Messphysik, Fuerstenfeld, AT) we completed displacement-driven, large-strain, multi-axial simple shear tests up to 25% shear strain using a quasi-static displacement rate (75 µm/min) under 1% axial compression on specimens of human articular cartilage.
4.2.1 Specimen preparation

Tissue arrived from the Musculoskeletal Transplant Foundation sealed in sterile buffered preservative solution (Penicillin G, Streptomycin sulfate, Polyoxyethylene phenol ether, NCTC Medium 135, Amphotericin B, Fetal calf serum, and DMEM) at 4°C. We harvested human cartilage from the lateral femoral condyles and patellofemoral grooves of six male donors (D1-6, 30.2 ± 8.8 yrs old, M±SD). We first determined the local SLD by pricking the articular surface with a dissecting needle (perpendicular to the surface) dipped in India ink, cf. Carballido-Gamio et al. (2008). Next we selected specimens from seven locations representing different loading conditions within the joint as shown in Fig. 4.1. Within the lateral condyle (LC) we selected three regions bearing load at different knee flexion angles: 0° (LC-L0), 75° (LC-L75) and 120° (LC-L120), and one non-load bearing region (LC-N), cf. Bingham et al. (2008). Within the patellofemoral groove (PFG) we selected locations to be in the center (PFG-C) and lateral side (PFG-L) of the joint, and at the border to the lateral condyle (PFG-B). We extracted our specimens close to the places where we determined the SLDs.

We then extracted square specimens (~3 × 3 mm², full thickness) using razor and scalpel blades while ensuring one edge of the specimen’s foot print was parallel to the local SLD. Finally, we removed all of the underlying trabecular bone and sufficient subchondral bone to create a surface parallel to the articular surface. We took care to ensure that the cartilage-bone interface remained intact, thus ensuring in-situ boundary conditions while testing. Using a digital precision caliper we measured the thickness of each cartilage specimen, excluding any subchondral bone, and took the average of five measurements. We immersed specimens not immediately tested in Phosphate Buffered Saline (PBS) and stored them at -80°C, cf. Szarko et al. (2010).

We generated histological images from tissues in representative regions near our specimens following a standard protocol using Safranin O/Fast Green (NovaUltra Special Stain Kits, IHC WORLD, Woodstock, MD). Using standard histological scoring we determined the
Figure 4.1: Specimen locations and orientations, and the local split-line direction (SLD), within the joint of Donor 2 from the (a) top, (b) anterior and (c) posterior view. Labeled squares mark specimen locations and orientations while protruding black lines indicate the local SLD. Red boxes denote the patellofemoral groove, located at the center (C), lateral side (L) and at the border to the lateral condyle (B). Blue boxes denote the lateral condyle, separated into regions bearing load at $0^\circ$ ($L_0$), $75^\circ$ ($L_{75}$), $120^\circ$ ($L_{120}$) of knee flexion, and non-load bearing (N). Letters outside the joint indicate medial (M), lateral (L), anterior (A) and posterior (P) orientations.

corresponding OARSI score of each specimen (Pritzker et al. 2006) and considered only healthy tissue with no signs of degeneration (OARSI score of $0 - 1$).

### 4.2.2 Triaxial shear test

We mounted specimens to two steel platens (covered with sandpaper) using cyanoacrylate gel. The top platen was rigidly connected to a triaxial load cell (K3D60, Me-Messsysteme, Henningsdorf, DE) mounted to a movable platform ($z$-direction), while the bottom platen was connected to a biaxial translation stage ($x$-, $y$-direction), cf. Sommer et al. (2015). The load cell had a resolution of 0.17 mN with a linear error $\leq 0.2\%$. The stroke resolution in the $x$- and $y$-direction was 0.25 $\mu$m and 0.04 $\mu$m in the $z$-direction. To ensure robust adhesion we compressed the fluid-saturated specimens under 0.2 N force for 200 sec while the glued dried. We then removed the compressive force, added PBS and allowed the load-free tissue to equilibrate for 2000 sec to compensate for possible fluid loss and to fully
recover the mechanical properties (Boettcher et al. 2016).

Prior to shear testing we applied an axial pre-compression of 1% of the undeformed thickness at a displacement rate of 10 \( \mu \text{m} / \text{min} \) and allowed the specimens to equilibrate for 4000 sec. Next we applied cyclic simple-shear displacements at a rate of 75 \( \mu \text{m} / \text{min} \), both parallel and perpendicular to the local SLD (six cycles per direction). We applied maximum displacements corresponding to shear strains of \( \pm 5\% \), \( \pm 10\% \), \( \pm 15\% \), \( \pm 20\% \) and \( \pm 25\% \) (with respect to the undeformed thickness) and measured the corresponding reaction forces in time. In between applied displacements parallel and perpendicular to the local SLD we allowed the specimens to equilibrate for 3000 sec. Additionally we randomized the direction of the first application of strain (parallel or perpendicular to the local SLD).

We completed all tests in a bath of PBS at 37 \( \pm 1^\circ \text{C} \) including antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) and protease inhibitors (Sigma Aldrich, St. Louis, MO) to avoid tissue degeneration.

### 4.2.3 Data analysis

We calculated the shear strain \( \gamma \) and corresponding shear stress \( \tau \) as

\[
\gamma = \frac{\Delta l}{L} \quad \text{and} \quad \tau = \frac{f}{A},
\]

where \( \Delta l \) is the applied displacement, \( L \) is the undeformed thickness of the specimen, \( f \) is the measured force and \( A \) the cross-section (foot print) of the specimen parallel to the measured force. For analysis we also calculated the peak-to-peak shear stresses and the strain-energy dissipation densities at each applied strain magnitude, both calculated by averaging over the last three loading cycles (to avoid preconditioning effects). We used MATLAB (The MathWorks Inc., Natick, MA) for all calculations, particularly we calculated the energy dissipation density using the MATLAB function \( \text{polyarea} \) (to determine
the area within the stress-strain curve).

4.2.4 Statistical analysis

First we checked if our results were normally distributed using a Shapiro-Wilk test. If shear stress, shear strain, energy dissipation density, sample thickness, or donor age were normally distribution we reported the mean and standard deviation (M±SD), or otherwise we reported the median and interquartile range. To probe anisotropy of tissue from both the patellofemoral groove and the lateral condyle we used the Wilcox rank sum test to compare mechanical responses parallel and perpendicular to the local SLD. We reported significant results as the differences in peak-to-peak shear stresses and strain-energy dissipation densities measured under shear applied parallel and perpendicular to the local SLD.

To probe inter- and intra-donor variability we conducted a Kruskal-Wallis test by ranks, with donor (D1-6), location (L1-7), and anatomical position (patellofemoral groove and lateral condyle) as categorical variables. Following significant correlations we conducted post-hoc analyses, using a Tukey test on the ranked results, to look for groupings within the categorical variables. We also assessed the influence of thickness and age using Pearson’s correlation coefficient \( r \).

We completed all statistical analyses using SAS 9.4 (SAS Institute Inc., Cary, NC) with a significance level of 0.05. Where informative we fit linear or nonlinear trend lines using the nonlinear least-square solver lsqcurvefit in MATLAB.

4.3 Results

We completed a total of 420 shear tests using 42 healthy cartilage specimens from six male donors.

We show a representative shear stress-strain plot from Donor 3, Location 3 in Fig. 4.2. We can observe a nonlinear stress-strain relationship with distinct hysteresis for all magnitudes
of applied strain.

![Figure 4.2: Representative shear stress-strain plot from Donor 3, Location 3: (a) parallel and (b) perpendicular to the local split-line direction.](image)

Peak-to-peak shear stresses and strain-energy dissipation densities at each applied strain magnitude and across joint locations were not normally distributed. Correspondingly, we analyzed all stress and energy results using tools for non-normally distributed data sets for consistency (as described above). Conversely both specimen thickness and donor age followed normal distributions.

Ten total cartilage specimens (five from each the patellofemoral groove and lateral condyle) showed signs of mechanical failure at 25%, i.e. an instant drop in the measured force response, and we excluded these data from our analyses.

### 4.3.1 Mechanical anisotropy

We show scatter plots of the complete mechanical data in Fig. 4.3. The difference in peak-to-peak shear stresses and strain-energy dissipation densities measured under shear applied parallel and perpendicular to the local SLD was significant under all applied strains (5 - 25 %) within the patellofemoral groove. This trend is evident in Fig. 4.3(b) and (d) where the majority of data points is below the line indicating isotropy. Within the lateral condyle a trend is less evident qualitatively (Fig. 4.3(a) and (c)).
Table 4.1: Values of significant differences in peak-to-peak shear stresses ($\Delta \tau = \tau_\parallel - \tau_\perp$) and strain-energy dissipation densities ($\Delta E = E_\parallel - E_\perp$) measured under shear strains $\gamma$ applied parallel and perpendicular to the local split-line direction given as median and interquartile range († indicates non-significant correlation, $p$-value = 0.06).

<table>
<thead>
<tr>
<th>$\gamma$ (%)</th>
<th>Patellofemoral Groove</th>
<th>L. Condyle</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta \tau$ (kPa)</td>
<td>12.57 (20.0, 1.51)</td>
<td>22.14 (33.8, 8.26)</td>
</tr>
<tr>
<td></td>
<td>27.13 (41.0, 19.6)</td>
<td>28.69 (48.1, 20.1)</td>
</tr>
<tr>
<td></td>
<td>25.69 (50.7, 15.5)</td>
<td>26.01 (38.2, 8.1)</td>
</tr>
<tr>
<td></td>
<td>17.68 (38.2, 8.1)</td>
<td>26.97 (45.0, 11.4)</td>
</tr>
<tr>
<td>$\Delta E$ ($\mu$N/mm$^3$)</td>
<td>0.165 (0.2, 0.1)</td>
<td>0.625 (1.1, 0.2)</td>
</tr>
<tr>
<td></td>
<td>1.31 (2.2, 0.6)</td>
<td>1.995 (3.8, 1.1)</td>
</tr>
<tr>
<td></td>
<td>3.09 (4.3, 1.5)</td>
<td>1.055 (2.6, 0.1)</td>
</tr>
<tr>
<td></td>
<td>0.82† (3.5, -0.3)</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis confirmed significant differences in peak-to-peak shear stresses ($\Delta \tau$) and strain-energy dissipation densities ($\Delta E$) under all applied strains (5 - 25 %) in the patellofemoral groove and at large strains (20 - 25 %) in the lateral condyle. Results are summarized in Table 4.1. If anisotropic, cartilage was stiffer and dissipated more energy under shear deformations parallel to the local SLD as opposed to perpendicular to it. The difference in dissipated strain-energy (parallel versus perpendicular to the local SLD) was $\sim$10% of the average dissipated energy at each strain level. The corresponding relative difference in peak-to-peak shear stresses decreased with increasing strain.

To probe the relationship between shear stress and energy dissipation further we show the relationship between strain-energy dissipation densities and peak-to-peak shear stresses in Fig. 4.4. Therein, the energy dissipation appeared almost constant at the lowest strain magnitude (5%). With increasing strain magnitude the dissipated energy increases overly proportional to the peak-to-peak stress, indicated as nonlinear trend line.

### 4.3.2 Intra- and inter-donor variability

The Kruskal-Wallis test revealed significant differences among joint locations, i.e. mechanical heterogeneity. We show the peak-to-peak shear stresses and strain-energy dissipation densities summarized across all six donors per location in Fig. 4.5. We report the corresponding quantitative data in tabular form in Appendix A.

The Tukey test on data from the lateral condyle showed no significant difference among
Figure 4.3: Scatter plots of (a),(b) the peak-to-peak shear stresses and (c),(d) the strain-energy dissipation densities. Plots (a),(c) correspond to the lateral condyle and (b),(d) correspond to the patellofemoral groove. The line plotted at 45° corresponds to an isotropic material response while points below the line indicate greater stiffness in the local split-line direction.
Figure 4.4: Scatter plots of strain-energy dissipation densities versus peak-to-peak shear stresses for (a),(b) the mechanical response parallel to the local split-line direction (SLD) and (c),(d) the mechanical response perpendicular to the local split-line direction (SLD). Plots (a),(c) correspond to the lateral condyle and (b),(d) correspond to the patellofemoral groove. Trend lines correspond to nonlinear least-squares best fit.
Figure 4.5: Box plots at all strain levels of (a),(b) the peak-to-peak shear stresses and (c),(d) the strain-energy dissipation densities. Plots (a),(c) correspond to the mechanical response parallel to the local split-line direction (SLD) and (b),(d) correspond to the mechanical response perpendicular to the local SLD.
locations at strain magnitudes of 5% and 10%. At strain magnitudes 15 – 25% specimens from the load bearing region at 0° knee flexion angle (LC-L0) were significantly stiffer and dissipated more energy than specimens from the meniscus covered region (LC-N), both parallel and perpendicular to the local SLD. Within the patellofemoral groove specimens from the lateral side of the joint (PFG-L) differed significantly from the center (PFG-C) and near the intra joint boundary (PFG-B), showing higher stresses and higher energy dissipation perpendicular to the SLD at all strain levels. In the PFG-L stresses parallel to the local SLD were significantly higher than from both the PFG-C and PFG-B only at higher strain magnitudes (15, 20, 20%) while the energy dissipation showed no dependence on location.

The Kruskal-Wallis test revealed no significant differences between donors and joints.

4.3.3 Influence of thickness and age

We found no significant difference in the measured thicknesses of the specimens among donors. We show the donor-averaged specimen thickness by location in Fig. 4.6(c). We show scatter plots of the peak-to-peak shear stresses and strain-energy dissipation densities versus specimen thickness in Fig. 4.6(a),(b) and (d).

The magnitude of stress showed a moderate negative correlation coefficient r with thickness, beginning at 15% strain, parallel \( r_{15} = -0.36, r_{20} = -0.41, r_{25} = -0.46 \), where the subscript indicates the percent applied strain, as well as perpendicular \( r_{15} = -0.42, r_{20} = -0.51, r_{25} = -0.48 \) to the local SLD. Strain-energy dissipation density perpendicular to the local SLD also showed a moderate negative correlation with thickness starting at 10% strain \( r_{10} = -0.30, r_{15} = -0.33, r_{20} = -0.4, r_{25} = -0.41 \).

We found no correlations among our mechanical data and age.
Figure 4.6: Mechanical results in light of the specimen thicknesses: (a) and (b) the peak-to-peak shear stresses parallel and perpendicular to the local SLD respectively. (c) donor-averaged specimen thickness by location and (d) the strain-energy dissipation density perpendicular to the local SLD. All stress and energy plots include best-fit linear trend lines for each applied strain that correlated significantly with specimen thickness \( t \).
4.4 Discussion

We were the first to probe the mechanical anisotropy of human articular cartilage undergoing large shear strains (up to 25%). A similar experimental device (for triaxial shear testing) was first described by Dokos et al. (2000), and was recently applied to human heart tissues (Sommer et al. 2015) and to human arterial tissues (Sommer et al. 2016). We did freeze specimens prior to testing but it has been shown that freeze-thaw treatment does not alter the mechanical properties of articular cartilage, cf. Szarko et al. (2010).

Cartilage stiffness in both tension and compression is rate dependent but reaches a plateau if the applied deformation rate is sufficiently slow (Huang et al. 2001, 2003). In this case the mechanical response is intrinsic to the solid constituents, i.e. collagen and proteoglycans, and thus independent of the fluid flow. In preliminary studies we determined the rate required (75 \( \mu \text{m/min} \)) to ensure quasi-static loading such that our applied displacement rate had no effect on the stress-strain response, while increasing it caused stiffening and an increased energy dissipation. Thus, we neglect the effects of fluid flow on dissipation and assume the strain-energy dissipation reported originates from the intrinsic viscoelastic properties of the collagen and proteoglycan. The greatest testing frequency (at the lowest strain magnitude) was on the order of 0.01 Hz, consistent with the lowest testing frequencies reported in the cartilage mechanics literature, cf. Zhu et al. (1993), Buckley et al. (2013).

Our results, represented in Fig. 4.2, show a nonlinear stress-strain relationship with distinct hysteresis for all magnitudes of applied strain. This is consistent with the well-established theory that collagen fibers are the main load bearing component in articular cartilage under shear deformation (Zhu et al. 1993, Silverberg et al. 2014, Griffin et al. 2014). The non-linearity originates from (i) transition from bending to stretching dominated loading of the fiber network (Buckley et al. 2008, Feng et al. 2016) and, (ii) nonlinear stiffening of the network of fibers undergoing large strains, cf. Zopf et al. (2015).
Stiffening (the slope of the stress-strain curve near peak loading) of the tissue was more pronounced at 5% and 10% applied shear strain than at higher strains. Strain softening can be caused by reversible adaptation of the solid matrix to external loads via rearranging of the network of collagen fibers or collagen-collagen and collagen-proteoglycan cross-links within the matrix, but could also indicate irreversible damage. Glaser and Putz (2002) showed that the fiber network recovers after load removal (below a damage threshold) and thus we assume that equilibrating the specimens prior to each application of shear displacement removes the reversible (non-damage) effects.

We selected the lower bound (5%) to be in the upper range of previous studies where usually strains of 1 - 3% were applied, cf. (Simon et al. 1982) At applied shear strains of 25% ten specimens showed severe signs of tissue failure, a finding consistent with the experiments on bovine tissue by Motavalli et al. (2013). Our preliminary studies further suggested consistent tissue failure at shear strains higher than 25%, thus we set this as our upper bound. Tissue failure occurred with equal frequency in the patellofemoral groove and lateral condyle.

### 4.4.1 Mechanical anisotropy

To probe mechanical anisotropy we randomized the first application of strain parallel or perpendicular to the local SLD to avoid possible directionally systematic strain softening. The tissue’s anisotropy likely originates from recruitment of preferentially aligned collagen fibers. Differences in the symmetries of the mechanical responses (between the LC and PFG) under large shear are likely due to different distributions of fibers in the reference configurations and subsequent recruitment of preferentially aligned collagen fibers. The LC appears isotropic at strains up to 15% before becoming significantly anisotropic (Fig. 4.3). Motavalli et al. (2014) recently showed that in mature bovine cartilage under bulk shear the MZ and DZ underwent larger shear strain magnitudes than the SZ. We hypothesize that the preferential alignment in fiber orientations is less distinct in the LC than
in the PFG. For (nearly) isotropic fiber distributions, a mix of tensile and compressive principal normal strains (as seen in simple shear) may generate strain-induced anisotropy in the MZ of the LC Ateshian (2006). Mechanical anisotropy of the SZ alone is likely to be significantly more pronounced (Robinson et al. 2016).

4.4.2 Intra- and inter-donor variability

We selected locations within the joint which undergo different magnitudes of in vivo loading and which also ensured relatively homogeneous thicknesses within specimens (Carballido-Gamio et al. 2008). We found a significant strain-dependent difference between the overall mechanical properties of the lateral femoral condyle and patellofemoral groove depending on the specimen’s location.

The loading in the LC and PFG are clearly different (Swann and Seedhom 1993, Salzmann et al. 2011). Contact between the patella and patello-femoral groove is dominated by sliding and, to a lesser extent, small tilting and rotation of the patella relative to the groove (Nha et al. 2008, Katchburian et al. 2003). The tibiofemoral joint experiences a variety of loading modes in vivo, including axial compression, rolling, sliding, axial and vargus-vagus rotation (Simon et al. 1982, Blankevoort et al. 1991, Liu et al. 2010).

A common hypothesis found in the literature is that cartilage remodels in response to mechanical loads, e.g. collagen fiber alignment in the SZ appears to remodel to optimize the tensile properties under in vivo compression and movement (Herbage et al. 1972, Below et al. 2002, Wu and Herzog 2002). Additionally, Moger et al. (2007) reported variations in collagen fiber orientation in the DZ of equine metacarpophalangeal joints. Throughout the joint, fibers in the deep zones were generally aligned perpendicular to the articular surface. However, regions subjected to higher loads showed a higher degree coherency in fiber orientation, highlighting the possibility for a preferred fiber direction.

Within the patellofemoral groove tissue from the lateral side (PFG-L) was significantly stiffer and dissipated more energy under shear strains applied perpendicular to the SLD
relative to the center and close to the inter joint border (PFG-C and -B). We found the same pattern of significance for the peak-to-peak stress under strains applied parallel to the SLD, but only at larger strains. This pattern in mechanical behavior is consistent with findings from indentation tests by Garcia-Seco et al. (2005). For comparison Franz et al. (2001) reported that cartilage from the patellofemoral groove was softer than that from the condyle while Athanasiou et al. (1991) reported the opposite. Such findings suggest that mechanical variations within individual joints may exceed differences among joints since both results can be matched with our data by matching locations of interest.

At shear strains larger than 15% load bearing region LC-$L_0$ was significantly stiffer and dissipated more energy than the non load bearing region (LC-N), while specimens from the other load bearing regions LC-$L_{75}$ and LC-$L_{120}$ were somewhere in between. Measurements of the exact loading cartilage experiences \textit{in vivo} are currently limited to either investigations of single loading modes (experiments with few repetitions) or simulations (Blankevoort et al. 1991, Carballido-Gamio et al. 2008, Pitikakis et al. 2015, Sutter et al. 2015, Song et al. 2014).

The mechanical response in the LC was statistically indistinguishable at lower applied shear strains (Fig. 4.5), consistent with Silverberg et al. (2013), where differences were not evident in the bulk properties but only near the SZ. This likely indicates that the deformations were not sufficiently large to significantly recruit collagen fibers to bear load in tension.

Looking at both the lateral condyle and patellofemoral groove undergoing 5% applied strain the dissipation of strain energy appears almost constant over all locations, as indicated by the small box heights in Fig. 4.5 at 5%. The corresponding peak-to-peak stresses showed more variability. With increasing applied shear strain the strain-energy dissipation density increased nonlinearly with respect to increasing peak-to-peak stress, indicated by the trend lines in Fig. 4.4. This effect indicates that the mechanisms of strain-energy dissipation, which include mainly interactions of proteoglycan molecules, are decoupled
from the main load bearing mechanisms (which are dominated by the network of collagen fibers) and are more sensitive to the applied displacement.

No significant difference was found between the overall mechanical properties of patellar groove and lateral femoral condyle across donors, and thus intra- exceeds inter-donor variability.

### 4.4.3 Influence of thickness and age

Since the patellofemoral groove and lateral condyle showed no significant differences we combined them to improve our search for significant correlations among our test parameters.

Our measured thicknesses, shown in Fig. 4.6(c), are in the same range as those reported via *in vivo* MRI measurements, cf. Carballido-Gamio et al. (2008) and Pitikakis et al. (2015). We found no significant correlations between our mechanical responses measured at 5% applied strain and thickness, a finding consistent with Silverberg et al. (2013). However, under larger shear strains (15 – 25%) articular cartilage showed significantly reduced stiffness with increasing thickness (in directions both parallel and perpendicular to the local SLD), and significantly reduced strain-energy dissipated under strains applied perpendicular to the local SLD (Table 4.6). Interestingly, the amount of strain-energy dissipated under shear applied parallel to the local SLD did not correlate significantly with specimen thickness. Such lack of correlation could indicate that strain-energy dissipation under shear applied parallel to the local SLD is governed by mechanisms within the SZ. Espino et al. (2014) showed that while storage and loss modulus decreased with cartilage thickness the ratio of those two measurements (an indicator for energy stored within the tissue) was insensitive to changes in specimen thickness. Direct comparison between these results and those presented here is difficult due to differences in the mechanical tests.

The lack of correlation between specimen thickness and age can be explained by the narrow age range of our donors (30.2 ± 8.82 yrs old), consistent with Blazek et al. (2014).
This also explains why we found no significant correlations between age and any of our mechanical measurements, our tissue specimens were all from a relatively narrow range of skeletally mature donors. Additionally, we verified the healthy condition of our specimens, and thus the associated integrity of the collagen and proteoglycan matrix that governs the mechanical responses (Silverberg et al. 2014).

### 4.4.4 Limitations and outlook

Axial compression during shear deformation alters cartilage’s shear response by altering the mechanical environment of the collagen fibers, and thus the amount of shear strain needed to recruit collagen fibers to bear load in tension. However, there are apparently conflicting results in the literature. Buckley et al. (2008) reported that the shear stiffness of cartilage is reduced with axial compression, while both Zhu et al. (1993) and Nguyen et al. (2010) reported the opposite. This apparent contradiction may originate from the different testing protocols (simple vs. torsional shear) used in the experiments, or changes in the geometry of the specimens that may have been neglected (e.g. ‘barreling’) during compression. In our test protocol the bonded connection between the specimens and the loading platens eliminated the need to apply axial compression to generate sufficient friction to transmit applied shear displacements (Motavalli et al. 2014). We thus applied 1% axial compression to provide baseline results with an unaltered cross-section and distribution of constituents. In future studies we will address this open question using digital image correlation over the full dimensions of the specimens to assess heterogeneous changes in geometry.

Here the applied displacement rate beyond 5% applied shear strain was non-physiological (less than 1 Hz). Further investigations should expand the understanding of cartilage mechanics under large shear strains to *in vivo* loading conditions. The tissue’s mechanical responses *in vivo* depend also on the permeation of interstitial fluid, which in turn depends on the rate of loading and the orientation of collagen fibers (Meder et al. 2006, de Visser
et al. 2008b). By virtue of our test protocol these mechanisms, which likely impact the anisotropic response of the tissue, were not investigated.

The bulk shear strain values applied in our study represent the average shear displacement within the specimens. The heterogeneous structure and constitution of cartilage causes strong through-the-thickness variations in the mechanical properties and hence responses. Application of digital image correlation (DIC) in combination with Ex-vivo mechanical experiments in both compression (Schinagl et al. 1997, Wang et al. 2002, Gannon et al. 2015) and shear (Wong et al. 2008a, Buckley et al. 2008, 2010, 2013, Motavalli et al. 2014) revealed heterogeneous strain fields with the highest strain magnitudes typically near the articular surface. DIC enlightens our understanding of the tissue’s structure-function relationships, both most experimental setups to date allow analysis of only one surface. DIC experiments will need to be expanded to allow visualization of multi-axial deformations aimed at understanding zonal variations in the tissue’s anisotropy.

We did not attempt to determine bulk material parameters such as the dynamic shear modulus since we violated the underlying assumption of geometric linearity, i.e. small deformations (Mow et al. 2005).

Our results suggest that full understanding of cartilage mechanics requires large-strain analyses to account for nonlinear, anisotropic and location-dependent effects not present at small strains. Shear stresses in cartilage specimens from the patellofemoral grooves were higher, and more energy was dissipated, at all applied strains under loading parallel to the local SLD versus perpendicular, while specimens from the lateral condyles were mechanically anisotropic only under larger strains of 20% and 25%. Cartilage also showed significant intra-donor variability at larger shear strains but no significant inter-donor variability.
Table 4.2: Peak-to-peak shear stress magnitudes parallel (∥) and perpendicular (⊥) to the local split-line direction given as median and interquartile range.

<table>
<thead>
<tr>
<th></th>
<th>Patellofemoral Groove</th>
<th>Lateral Condyle</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ (%)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>τ∥ (kPa)</td>
<td>58.36</td>
</tr>
<tr>
<td></td>
<td>[77.04,103.8]</td>
<td>[75.63,113.5]</td>
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<td></td>
<td>τ⊥ (kPa)</td>
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</tr>
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<td>10</td>
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<td></td>
<td>τ⊥ (kPa)</td>
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Table 4.2: Peak-to-peak shear stress magnitudes parallel (∥) and perpendicular (⊥) to the local split-line direction given as median and interquartile range.

Appendix P1

We report the quantitative data for the peak-to-peak shear stresses in Table 4.2 and for shear strain-energy dissipation density in Table 4.3.
### Table 4.3: Shear strain-energy dissipation parallel (∥) and perpendicular (⊥) to the local split-line direction given as median and interquartile range.

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<th>γ (%)</th>
<th>Patellofemoral Groove</th>
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<td>1.34 [1.23,1.44]</td>
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<td>$E_\parallel$ (mJ/mm$^3$)</td>
<td>1.65 [1.56,1.80]</td>
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<td>$E_\perp$ (mJ/mm$^3$)</td>
<td>1.56 [1.41,2.06]</td>
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<td>$E_\perp$ (mJ/mm$^3$)</td>
<td>1.60 [1.58,2.29]</td>
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<td>$E_\perp$ (mJ/mm$^3$)</td>
<td>1.44 [1.30,1.50]</td>
</tr>
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<td>4.39 [3.32,4.90]</td>
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<td>$E_\parallel$ (mJ/mm$^3$)</td>
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<td>$E_\perp$ (mJ/mm$^3$)</td>
<td>5.26 [4.38,5.36]</td>
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<td>$E_\perp$ (mJ/mm$^3$)</td>
<td>28.7 [24.3,33.9]</td>
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<td>$E_\perp$ (mJ/mm$^3$)</td>
<td>34.0 [31.2,44.0]</td>
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<td>$E_\perp$ (mJ/mm$^3$)</td>
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<td>$E_\perp$ (mJ/mm$^3$)</td>
<td>19.5 [16.0,20.5]</td>
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5 Evolving Large-Strain Shear Responses of Progressively OA Human Cartilage

5.1 Introduction

Osteoarthritis (OA) initiates with deaggregation of glycosaminoglycans (GAGs), increased osmotic pressure (Buckwalter et al. 2005), and softening of the collagen network, leading to increased water content, i.e. swelling and thus increased thickness (Adams and Brandt 1991). Subsequently, chondrocytes “activate” and increase production of both matrix proteins and matrix degrading enzymes (Goldring 2000, Roach et al. 2005). These changes in the mechanics of the tissue eventually exacerbate initial SZ collagen fibrillation into surface fissures, matrix loss, and erosion (Goldring and Goldring 2010). The permeability of cartilage increases in OA, subsequently reducing the tissue’s ability to generate and maintain hydrostatic pressure under load (Setton et al. 1995, Hwang et al. 2008). Furthermore, OA cartilage presents changes in zonal thicknesses and fiber orientations visible with both polarized light microscopy (Changoor et al. 2011b) and small angle X-ray scattering (Moger et al. 2007).

Such changes to the tissue’s composition and structure also lead to altered mechanical responses typical of OA. Franz et al. (2001) and Garcia-Seco et al. (2005) showed changes
in both structural integrity and composition correlated (respectively) with cartilage softening measured by indentation. Similarly, Robinson et al. (2016) demonstrated that cartilage under large strain compression (up to 30%) softens with the onset of OA. These studies used loading rates resembling those in vivo, where poroelastic effects (i.e. pressure in the interstitial fluid) dominate the mechanical responses, and did not investigate the decoupled mechanical response of the solid constituents.

Any mechanical changes in cartilage might be exploited as biomechanical markers for detection of OA. For human OA cartilage, Kleemann et al. (2005) showed the equilibrium modulus, measured in unconfined compression, reduced with increasing degeneration, a significant difference for severe late-stage OA versus healthy control tissue, but not for mild OA (ICRS grade 1) versus healthy control (ICRS grade 0). Similarly, Kumar et al. (2018) showed the parameters resulting from a time-dependent viscoelastic model fitted to indentation tests on progressively osteoarthritic cartilage correlated with degeneration, but also could not resolve the early stages of OA. In both studies the use of ICRS grading to quantify the progression of OA may have contributed to lack of sensitivity in detecting early-stage OA.

Chan et al. (2016) experimentally demonstrated that shear strains (approaching 12%) generally exceeded compressive strains under in-vivo compression, highlighting the need to understand cartilage mechanics in large-strain shear; however, few studies have investigated degraded or diseased cartilage under shear. Only Wong et al. (2008a) tested human OA cartilage under large-strain shear and reported increased strains near the articular surface. This study focused on the frictional properties of OA cartilage and restricted the articular surface using only friction of the opposing surface, making the applied deformation difficult to control and perhaps affecting the measured bulk shear properties.

There is a clear need to investigate the evolution of large-strain cartilage mechanics associated with early-stage OA to 1) better understand disease initiation and progression (to identify possible treatment targets), 2) identify potential biomarkers for OA diagnosis (to
test therapeutics), and 3) calibrate and validate advanced constitutive models (to better predict the initiation and progression of OA (Hosseini et al. 2014)).

In this study we quantify the evolution of the intrinsic, large-strain mechanics of the solid constituents of human articular cartilage, i.e. collagen and proteoglycan and their interactions (Hayes and Mockros 1971, Zhu et al. 1993), during the progression of OA as determined by OARSI grading. We correlate the evolution of measured bulk mechanical properties with measures of disease progression, structural integrity, and cartilage composition, and compare against results from healthy control cartilage (previously published in Maier et al. (2017)) for context.

5.2 Materials and Methods

Using a triaxial shear testing device (Messphysik, Fuerstenfeld, AT) we completed displacement-driven, large-strain tests on 64 specimens of progressively osteoarthritic human articular cartilage (following Maier et al. (2017)) as determined by both the OARSI grade and the PLM-CO score.

5.2.1 Preparation of specimens

We harvested ten lateral femoral condyles (three male and seven female, aged 65.8±12.5 years) from patients undergoing total knee arthroplasty (TKA) at Hartford Healthcare Bone & Joint Institute and transported them, submerged in phosphate-buffered saline (PBS), to our lab within eight hours of extraction. We first determined the split-line direction (SLD, cf. Below et al. (2002)) by pricking the articular surface with a needle dipped in india ink. We then extracted pairs of specimens from various locations across the donor joint – one for histological assessment (Section 5.2.2) and quantification of constituents (Section 5.2.3) and the second, directly adjacent, for mechanical testing (cubiod, $3 \times 3 \text{ mm}^2$ footprint, full thickness and with one edge aligned parallel to the local SLD if distinctly present;
Section 5.2.4). For specimens dedicated to mechanical testing we carefully removed the underlying trabecular bone and sufficient subchondral bone both to create a surface parallel to the articular surface and to ensure the intact cartilage-bone interface mimicked the in-situ boundary conditions while testing (Morel and Quinn 2004). We stored these specimens submerged in PBS at $-80^\circ$ until mechanical testing. We fixed the specimens for histological assessment in 10% neutral buffered formalin.

5.2.2 Histological assessment

After decalcification in 0.5 M EDTA for one week, we embedded the specimen in paraffin and sectioned at 6 $\mu$m. To quantify overall cartilage health, we stained slides with Safranin-O fast green (Novaultra Safranin O stain kit, IHC World, Ellicott City, MD) and then applied the OARSI grading method (Pritzker et al. 2006). Two independent, trained observers (FM, DP) determined the local OARSI grade, and we considered specimens ranging from OARSI grade 1 (normal) to 4.5 (severe disease) for mechanical testing. We binned the specimens into four groups based on their OARSI grade: normal (OA-1, grade < 2), mild (OA-2, 2 $\leq$ grade < 3), moderate (OA-3, 3 $\leq$ grade < 4), and severe (OA-4, grade $\geq$ 4) OA.

To assess the integrity of the zonal architecture, i.e. heterogeneity in the network of collagen fibers, we stained additional slides with Picosirius red (Novaultra Sirius red stain kit, IHC World) and examined these under polarized light. Two independent, trained observers (FM, LM) quantified the zonal architecture using the PLM-CO score (Changoor et al. 2011a). This score uses an ordinal scale, ranging from disorganized (score 0) to that resembling a healthy control zonal architecture (score 5). We binned the specimens into two groups based on their PLM-CO score: specimens with evident zones (EZ, PLM-CO $\geq$ 3) and specimens without (NZ, PLM-CO < 3).

If necessary to image an entire specimen, we acquired multiple images and stitched them together using the MosaicJ plugin for ImageJ (1.51n, National Institutes of Health, Bethesda,
5.2.3 Quantification of constituents

To quantify the mass fractions of the constituents, we sectioned cartilage specimens fixed for long-term storage, and we then deparaffinized and dried the specimens, removed any subchondral bone, and measured their dry weights. We then rehydrated the specimens with a decreasing alcohol series to PBS (van Wijk et al. 2012), and measured the wet weight. We completed digestion and analyses using a Glycosaminoglycan Assay Kit (6022, Chondrex, Redmond, WA) and a Hydroxyproline Assay Kit (6017, Chondrex). For analyses we used approximately 10 mg of tissue, solubilized in 1.25 ml digestive solution (125 µg/ml Papain (60224, Chondrex) in PBS at pH 6.3 with 5 mM L-cystein-HCL and 10 mM EDTA-2Na), incubated at 65°C for 36 hours. We then centrifuged this solution at 10,000 rpm for five minutes and transferred the supernatant for analyses with the GAG assay. After determining the GAG concentration, we hydrolyzed 100 µl of the dissolved tissue in 10 N hydrochloric acid and quantified hydroxyproline concentration. We multiplied this concentration by 7.4 to get total collagen (following the protocol from Chondrex). We reported all concentrations per wet and dry weight.

5.2.4 Triaxial shear test

We used our testing device and protocol as previously described in Maier et al. (2017). Briefly, we performed quasi-static (75 µm/min) cyclic simple shear tests, both parallel and perpendicular to the local SLD (when present), on cartilage specimens under 1% precompression. To improve adherence, we glued specimens to polymethyl methacrylate (PMMA) platens which we glued to stainless steel platens using cyanoacrylate gel. At each phase of the test we allowed specimens to equilibrate, i.e. after gluing, after applying precompression, and when changing the loading direction for 2,000, 4,000, and 600 seconds respec-
tively. We applied maximum displacements corresponding to shear strains of ±5%, ±10%, ±15%, and ±20% (with respect to the undeformed thickness) for six cycles per direction and measured the corresponding reaction forces in time. We completed all tests in a bath of PBS at 37 ± 1°C including antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) and protease inhibitors (Sigma Aldrich, St. Louis, MO) to avoid tissue degeneration.

5.2.5 Data analyses

We calculated the shear strain γ and corresponding shear stress τ as

$$\gamma = \frac{\Delta l}{L} \quad \text{and} \quad \tau = \frac{f}{A},$$

(5.1)

where $\Delta l$ is the applied displacement, $L$ is the undeformed thickness of the specimen, $f$ is the measured force, and $A$ is the cross-section (footprint) of the specimen parallel to the measured force. For subsequent analyses we calculated the strain-energy dissipation density ($E_{DI}$) and the peak-to-peak shear stress ($\tau_{PP}$) at each applied strain magnitude as

$$E_{DI} = \int_{\gamma_{min}}^{\gamma_{max}} \tau \, d\gamma,$$

(5.2)

and

$$\tau_{PP} = \tau(\gamma_{max}) - \tau(\gamma_{min}),$$

(5.3)

where $\gamma_{max}$ and $\gamma_{min}$ are the maximum and minimum shear strain of a given loading cycle. We also calculated the peak effective shear modulus ($G_{PE}$) by progressively finding the slopes of linear least-squares fits over ten progressive data points along our stress-strain loading curves (Santos et al. 2017). For each applied strain magnitude, we determined
both the positive and negative slopes as

\[
G^{+/-} = \frac{\tau_{ave}}{\gamma_{ave}}, \quad \text{where} \begin{cases} 
  & \text{for } +, \tau_{ave} \geq 0 \text{ and } \gamma_{ave} \geq 0 \\
  & \text{for } -, \tau_{ave} < 0 \text{ and } \gamma_{ave} < 0 
\end{cases}
\]

for all linear fits along each loading curve, and where \(\tau_{ave}\) is the vertical projection of the best-fit line and \(\gamma_{ave}\) is the complementary horizontal projection. After determining the maximum positive and negative slopes, \(G^{+}_{\max}\) and \(G^{-}_{\max}\), we averaged them to obtain the peak effective shear modulus \(G_{PE}\) at each strain magnitude. Figure 5.1 illustrates our mechanical data analyses. In our tests the force responses were repeatable after three preconditioning cycles, and we averaged all calculated values over the next three loading cycles. We completed all calculations using MATLAB R2017a (The MathWorks Inc., Natick, MA).

5.2.6 Statistical analyses

First, using a Shapiro-Wilk test, we confirmed that the outputs of our mechanical data analyses were normally distributed. To validate inter-observer agreement in our application
of the OARSI and PLM-CO grading methods we calculated the linear weighted Cohen’s kappa coefficient $\kappa$. Furthermore, we used an ordinal regression to see how OA (OARSI grade) affects structure (PLM score). To probe for anisotropy, we used the Wilcoxon Rank-Sum test to compare mechanical responses parallel and perpendicular to the local SLD. To probe for effects from progressing OA, we conducted Kruskal-Wallis tests on composition (proteoglycan and collagen content), structure (PLM-CO score, thickness), and mechanics ($E_{D1}$, $\tau_{PP}$, $G_{PE}$) of cartilage gathered from OA knees (OA-1 to -4).

To further assess influence of OA, we used data from young healthy control cartilage, published in our previous work (Maier et al. 2017), as a benchmark. Data on healthy control cartilage corresponds to load-bearing at $0^\circ$ knee flexion (HL$_0$, $n = 6$) and non-load bearing, i.e. covered by the menisci, (HNL, $n = 6$) regions. These data represent upper and lower bounds on healthy cartilage, as they are the stiffest and softest healthy adult cartilage from the lateral condyles.

When we identified significant correlations, we used pairwise comparisons (two-tailed Student’s $t$-test or Wilcoxon Rank-Sum test, depending on normality) to identify significant differences within OA groups (number of comparisons $m = 6$) and to compare OA groups to healthy controls ($m = 4$). We assessed the influence of proteoglycan and collagen content, age, and thickness on our mechanical metrics using Pearson’s (Spearman’s) correlation coefficient $r$ ($r_S$) ($m = 4$). We used $\alpha = 0.05$ to test for significance and adjusted this for our multiple comparison tests using the Holm-Bonferroni method ($\alpha = 0.05/m$).

We completed all statistical analyses using SAS 9.4 (SAS Institute Inc., Cary, NC).

### 5.3 Results

We completed a total of 512 shear tests using 64 specimens. We show representative shear stress-strain plots comparing specimens with OARSI grade 0 and OARSI grade 4 in Fig. 5.2. We observed nonlinear stress-strain relationships with distinct hystereses for
all magnitudes of applied strain where stiffness, nonlinearity, and hysteresis reduce with advancing OA.

Thirty of our cartilage specimens showed signs of mechanical failure during testing (two at 10%, six at 15%, and 23 at 20% applied strain), visible as an instant drop in the measured force response, and we excluded these data from our analyses. We provide a detailed summary relating each mechanical test (applied strain magnitude) to the patient number and OARSI grade in Table A3.

### 5.3.1 Histological assessment

We show representative images of Safranin-O stained histological slides and Picosirius-red stained slides under polarized light in Figs. 5.3 and 5.4, respectively.

Our OARSI grading resulted in $n_{OA-1} = 19$, $n_{OA-2} = 25$, $n_{OA-3} = 14$, and $n_{OA-4} = 6$ specimens per binned group. Intra-observer agreement was $\kappa = 0.81$ for unbinned grades and $\kappa = 0.97$ for binned grades. Our PLM-CO scoring resulted in $n_5 = 5$, $n_4 = 9$, $n_3 = 15$, $n_2 = 28$, and $n_1 = 4$, with $\kappa = 0.68$ (unbinned) and $\kappa = 0.92$ (binned). For our subsequent analyses we averaged the grades from both observers and assigned them to a bin (group), cf. Sec. 5.2.6. Normal cartilage specimens (OA-1) had significantly higher
5.3 Results

Figure 5.3: Representative histological slides stained with Safranin-O fast green for assessing the severity of osteoarthritis using the OARSI grading method: (a) OARSI grade 1 (OA-1) – intact surface and proteoglycan (PG) content, (b) OARSI grade 2 (OA-2) – increased surface roughness and loss of PG, (c) OARSI grade 3 (OA-3) – vertical fissures and greater loss of PG, and (d) OARSI grade 4 (OA-4) – severe surface disruption and loss of PG. PG stains red. Bars indicate 500 µm.

Figure 5.4: Representative histological slides stained with Picosirius red and visualized under polarized light for assessing the structural integrity using the PLM-CO scoring method: (a) PLM-CO score 5 – healthy zonal architecture, (b) PLM-CO score 4 – proportional but laterally heterogeneous zones, (c) PLM-CO score 3 – present but disrupted through-thickness proportions of zones, (d) PLM-CO score 2 – intact deep zone but missing other zones, and (e) PLM-CO score 1 – present but underdeveloped (<50% of thickness) deep zone. Bright yellow indicates areas with fibers aligned parallel to the articular surface, black indicates randomly isotropic alignment, and bright blue-green indicates areas with fibers aligned perpendicular to the surface. Bars indicate 500 µm.
PLM-CO scores versus progressively osteoarthritic specimens; see Fig. 5.5(a).

Figure 5.5: (a) Boxplots of the PLM-CO scores binned by OARSI grades show higher PLM-CO scores in normal cartilage versus those with progressively advanced osteoarthritis. (b) Boxplots of cartilage thicknesses binned by OARSI grades show no significant differences between groups. (c) GAG concentration in milligram per gram wet cartilage binned by OARSI grades. (d) Collagen concentration in milligram per gram wet cartilage binned by OARSI grades. Bars labeled * indicate significant differences between groups ($p < 0.05$). Red plus signs mark outliers calculated as $Q_3 \pm 1.5(Q_3 - Q_1)$, where $Q_1$ and $Q_3$ denote the 25th and 75th percentiles of the sample data, respectively.

We found no significant differences in cartilage thicknesses binned by OARSI grades and PLM-CO scores, and compared to healthy controls (not shown); see Fig. 5.5(b).
5.3.2 Quantification of constituents

GAG concentration per wet weight significantly reduced with increasing OARSI grade, i.e. degeneration ($r = -0.4858, p < 0.0001$); see Fig. 5.5(c). Our pairwise comparisons showed that normal cartilage (OA-1) presents a 17% ($p = 0.0085$) and 33% ($p = 0.0007$) higher GAG concentration per wet weight than moderate (OA-3) and severely degenerated (OA-4) cartilage, respectively, and that mildly degenerated tissue (OA-2) has a 25% ($p = 0.0092$) higher concentration than severely diseased (OA-4) cartilage, cf. Fig. 5.5(c). Total collagen concentration by wet weight showed no significant dependence on progressing OA, see Fig. 5.5(d).

GAG concentration per dry weight followed qualitatively the same trends as per wet weight, i.e. a significant reduction in dry weight with increasing OARSI grade ($r = -0.4250, p = 0.0006$). Conversely, collagen concentration per dry weight increased in dry weight with increasing OARSI grade ($r = 0.4250, p = 0.0006$).

5.3.3 Mechanical anisotropy

Data collected from specimens with a distinct SLD ($n = 52$) showed anisotropic responses in $E_{DI}$ at 5% ($p = 0.0029$), 10% ($p = 0.0425$), and 20% ($p = 0.0059$) applied strains, and $\tau_{PP}$ ($p = 0.0392$) and $G_{PE}$ ($p = 0.0044$) at 20% applied strain. If anisotropic, cartilage dissipated on average more energy and was stiffer in the SLD; see Table 5.1.

Data collected from specimens not presenting a distinct SLD ($n = 12$) did not show a dependence on direction.

In light of the mild anisotropy (if present), and to increase statistical power, we averaged the data from our two perpendicularly oriented shear tests and binned all of our data for subsequent analyses.
5.3.4 Degeneration

The Kruskal-Wallis test confirmed significant differences in mechanics among OARSI groups and versus our healthy benchmark data. We show $E_{DL}$, $\tau_{PP}$, and $G_{PE}$ summarized across all ten donors and binned by grades (including benchmarks) in Fig. 5.6(a-c).

Within the OA groups we found that severely degenerated cartilage (OA-4) dissipated less strain energy and showed reduced peak-to-peak stresses and reduced peak-effective shear moduli versus normal OA tissue (OA-1). Healthy (control) load-bearing cartilage (HL$_0$) dissipated significantly more strain energy versus any cartilage originating from a diseased knee (OA-1 to -4). Non-load-bearing cartilage (HN) dissipated more energy than mildly to severely degenerated cartilage (OA-2 and -4) at some applied strain levels. Peak-to-peak shear stresses $\tau_{PP}$ from healthy load-bearing cartilage (HL$_0$) were significantly higher versus those from mildly to severely degenerated cartilage (OA-2 and -4). Peak-to-peak shear stresses were not significantly different between healthy non-load-bearing cartilage (HN) and degenerated cartilage (any). Healthy load-bearing cartilage was only significantly stiffer ($G_{PE}$) than severely degenerated cartilage at larger applied strains (15-20%). Normal cartilage from an OA joint (OA-1) had a significantly stiffer response than severely degenerated cartilage (OA-4) as dissipated less strain energy and showed reduced peak-to-peak shear stresses and reduced peak-effective shear moduli versus normal OA tissue (OA-1).

Table 5.1: Values of significant differences in strain-energy dissipation densities ($\Delta E_{DL}$), peak-to-peak shear stresses ($\Delta \tau_{PP}$), and peak effective shear modulus ($\Delta G_{PE}$) measured at shear strains $\gamma$ applied both parallel and perpendicular to the local split-line direction given as predicted median and 95% confidence interval. The percent difference is $\frac{\Delta □_{median}(□⊥)}{□_{median}(□⊥)} \times 100$, where $median(□⊥)$ is the median value from data in the perpendicular direction.

<table>
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<th>20</th>
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<td>$\Delta E_{DL}$ (mJ/mm$^3$)</td>
<td>15.57 (0.0422,0.1763)</td>
<td>10.20 (0.02051,0.5377)</td>
<td>10.80 (0.02124,2.091)</td>
</tr>
<tr>
<td>CI</td>
<td>0.1099</td>
<td>0.2791</td>
<td>1.056</td>
</tr>
<tr>
<td>$p$</td>
<td>0.0019</td>
<td>0.035</td>
<td>0.0327</td>
</tr>
<tr>
<td>$\Delta \tau_{PP}$ (kPa)</td>
<td>-</td>
<td>-</td>
<td>14.74 (3.556,66.33)</td>
</tr>
<tr>
<td>CI</td>
<td>-</td>
<td>-</td>
<td>34.94 (5.56,66.33)</td>
</tr>
<tr>
<td>$p$</td>
<td>-</td>
<td>-</td>
<td>0.0308</td>
</tr>
<tr>
<td>$\Delta G_{PE}$ (kPa)</td>
<td>-</td>
<td>-</td>
<td>23.95 (88.13,419.7)</td>
</tr>
<tr>
<td>CI</td>
<td>-</td>
<td>-</td>
<td>253.9</td>
</tr>
<tr>
<td>$p$</td>
<td>-</td>
<td>-</td>
<td>0.0045</td>
</tr>
</tbody>
</table>
Figure 5.6: Box plots of the (a) strain-energy dissipation densities ($E_{DI}$), (b) peak-to-peak shear stresses ($\tau_{PP}$), and (c) peak effective shear moduli ($G_{PE}$) at all magnitudes of applied strain across all donors and binned by OARSI grades (OA-1 to -4), binned by PLM-CO scores ($\geq 3$ or $< 3$) (d-f). As a benchmark, we include data from load-bearing ($HL_0$) and non-load-bearing (HN) locations within healthy young joints (Maier et al. 2017). Bars labeled * indicate significant differences between groups ($p < 0.05$). The number of * indicates significance at that number of strain magnitudes, and we indicate the significant strain magnitudes, cf. Appendix A. Red plus signs mark outliers calculated as $Q_3 \pm 1.5(Q_3 - Q_1)$, where $Q_1$ and $Q_3$ denotes the 25th and 75th percentiles of the sample data, respectively.
degenerated cartilage at 5, 15, and 20% applied strain and was only stiffer than cartilage with mild (OA-2) and advanced degeneration at 5 and 15%, respectively. In Appendix P2, we report all the quantitative data for Fig. 5.6(a-c) in Table A4, and percent and absolute differences of shear strain-energy dissipations $E_{DI}$, peak-to-peak shear stresses $\tau_{PP}$, and peak effective shear moduli $G_{PE}$ in Tables A5 to A7.

5.3.5 Structural integrity

The Kruskal-Wallis test confirmed significant differences in mechanics among groups binned by PLM-CO score. We show $E_{DI}$, $\tau_{PP}$, and $G_{PE}$ summarized across all ten donors and binned by PLM-CO scores in Fig. 5.6(d-f). All mechanical measures ($E_{DI}$, $\tau_{PP}$, and $G_{PE}$) were 30-40% higher with the presence of zonal structure, consistent over all applied strain magnitudes. In Appendix P2, we report all the quantitative data for Fig. 5.6(d-f) in Table A4.

5.3.6 Collagen and proteoglycan content, thickness, and age

Total collagen content by wet weight, specimen thickness, and patient age (as independent factors) did not correlate with any mechanical measures ($E_{DI}$, $\tau_{PP}$, $G_{PE}$). All mechanical measures, except $E_{DI}$ at 5 and 10% applied shear strain, showed a weak to moderate correlation with GAG content; see Table 5.2. GAG concentration by dry weight shows qualitatively the same correlations with the respective mechanical measures as GAG by wet weight. While all mechanical measures increase with increasing GAG concentration, they similarly reduce with increasing collagen content. Thickness decreased significantly with age ($r = -0.7041, p < 0.0001$).
5.4 Discussion

We were the first to quantify the mechanical properties of progressively osteoarthritic human articular cartilage undergoing large shear strains (up to 20%). Cartilage shows a non-linear stress-strain relationship with a distinct hysteresis for all magnitudes of applied strain (cf. Fig. 5.2), where shear strain-energy dissipation, peak-to-peak shear stress, and peak effective shear modulus progressively reduce with advancing OA.

5.4.1 Histological assessment

We found good inter-observer agreement for OARSI grades and PLM-CO scores and had excellent agreement when comparing the binned groups (Sec. 5.2.6). In light of the heterogeneous intra-joint progression of OA, we used OARSI grade as a local measure of cartilage degeneration, versus the OARSI score, which also includes a joint-scale measure of OA. Utilizing the OARSI grading method also allowed us to resolve early stages of OA (Pritzker et al. 2006). Comparing our results to the literature is challenging due the use of different methods for quantifying the progression OA, e.g. Mankin scores or ICRS (Outerbridge) grades. Different quantification methods do show strong correlations, e.g. OARSI vs. Mankin (Pauli et al. 2012), Mankin vs. ICRS (Kleemann et al. 2005), but they differ in terminology and definitions of early-stage OA. For the subsequent comparisons, we interpret published results in the context of the OARSI grade.

At higher OARSI grades, finding specimens with consistent thicknesses—and thus with (reasonably) flat surfaces—became progressively more challenging. Hence, we have a smaller

<table>
<thead>
<tr>
<th>$\gamma$ (%)</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{DI}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>0.4342</td>
<td>0.4593</td>
<td>0.0016</td>
<td>0.0107</td>
</tr>
<tr>
<td>$p$</td>
<td>0.0001</td>
<td>0.0003</td>
<td>0.0003</td>
<td>0.0005</td>
</tr>
<tr>
<td>$\tau_{PP}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>0.4038</td>
<td>0.4541</td>
<td>0.4914</td>
<td>0.5973</td>
</tr>
<tr>
<td>$p$</td>
<td>0.0011</td>
<td>0.0003</td>
<td>0.0003</td>
<td>0.0005</td>
</tr>
<tr>
<td>$G_{PE}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>0.3717</td>
<td>0.3975</td>
<td>0.4034</td>
<td>0.5424</td>
</tr>
<tr>
<td>$p$</td>
<td>0.0029</td>
<td>0.0020</td>
<td>0.0037</td>
<td>0.0020</td>
</tr>
</tbody>
</table>

Table 5.2: Pearson’s correlation coefficient $r$ and $p$-values for significant correlations between our mechanical measures ($E_{DI}$, $\tau_{PP}$, $G_{PE}$) and GAG concentration.
sample number for our severely degenerated group (OA-4). However, the distinctly different mechanical responses still allowed us to determine significant differences. We determined the PLM-CO scores based on digital images, and if not clearly distinguishable, we directly observed slides under the microscope, better allowing us to rotate them. We oriented the slides shown in Fig. 5.4 to emphasize the main features for PLM-CO scoring. We did not post-process (i.e. enhance contrast or manipulate color) these PLM images, cf. Changoor et al. (2011a). Binning our PLM-CO scores by presence/absence of zonal structure, resulted in two groups of equal size ($\geq 3$ or $< 3$), improving statistical power and allowing us to investigate the general influence of collagen architecture. Our approach does not allow us to distinguish between different stages of structural degeneration.

### 5.4.2 Quantification of constituents

Both the GAG and total collagen content per wet weight we report are within ranges reported previously (Franz et al. 2001, Mow et al. 2005, Buckwalter et al. 2005), as is the progression of these with OA, i.e. a progressive reduction in GAG concentration and no significant change in collagen concentration (Temple-Wong et al. 2009). The combined weight of GAGs and collagen accounts for the strong majority of the total dry weight of cartilage (Mansour 2003, Mow and Huiskes 2005). Thus, statistics based on the constituents per dry weight does not allow independent observations on the effects of GAG and collagen concentrations, i.e. if GAG concentration reduces with degeneration, collagen concentration must increase. The concentration of water changes in cartilage as a function of OA (Buckwalter et al. 2005), thus concentrations of the constituents calculated by wet weight carry essential information regarding the integrity of cartilage. Fixation procedures likely cause collagen cross-linking and may compromise the capability of cartilage to rehydrate (Mekota and Vermehren 2005, Hunziker et al. 2014, Kansu et al. 2017). None-the-less all of our samples underwent the same treatment, i.e. fixation, decalcification, embedding, and storage; thus our results are self consistent.
5.4.3 Mechanical anisotropy

Shear strain energy dissipation correlated significantly with the SLD at all magnitudes of applied shear strain (except at 15%), indicating that dissipated energy is linked to organization of the collagen network. The mechanical measures $\tau_{PP}$ and $G_{PE}$ also correlated significantly with the SLD at larger strains (20%), similar to healthy control tissue (Maier et al. 2017). Quantifying mechanical anisotropy by OARSI grade (results not shown) did not reveal consistent trends. Anisotropy is more pronounced at the articular surface of healthy cartilage (Huang et al. 2005) due to the pronounced fiber alignment in the SZ. Using cartilage specimens tested in unconfined compression Robinson et al. (2016) reported a significant reduction in anisotropy near the articular surface with increasing degeneration. Our shear test may not reveal this change since we restrict the SZ in our experiment. Of the 12 specimens without a clear local SLD, five presented OARSI grade 2, five OARSI grade 4, and two OARSI grade 3, thus all showed signs of degeneration. However, this is not necessarily a sign of degeneration as even within healthy joints regions may show an isotropic fiber distribution, i.e. no apparent SLD (Silverberg et al. 2013).

5.4.4 Degeneration

We selected specimens from load-bearing regions of lateral condyles. We compared the mechanics of our OA specimens to the stiffest (HL0) and softest (HN) cartilage found in healthy lateral condyles, serving as an upper and lower bound to account for potential location-based differences.

Energy dissipation in cartilage stems from interactions of the fluid and solid phase, and from GAG-GAG and GAG-collagen interactions (Zhu et al. 1993). The quasi-static design of our experiment excludes fluid-solid interactions; thus our reported energy dissipation originates from solid-solid interactions only. We found a reduction in $E_{DI}$ up to 80% (cf. Table AA5) in severely degenerated (OA-4) versus normal (OA-1) cartilage, consistent...
with Abdel-Sayed et al. (2014a). Importantly, we found that even cartilage with a normal appearance in structure and composition (OA-1) dissipated \(\sim 50\%\) less energy than healthy (control) load-bearing cartilage (HL\(_0\)). In comparison to healthy non-load-bearing cartilage (HN), mildly (OA-2) and severely (OA-4) degenerated cartilage showed a strain-dependent reduction in dissipated energy of \(\sim 50\%\) and 70\% respectively. Furthermore, energy dissipation may play an important role in maintaining an optimum temperature for chondrocytes and chondrogenic expression (Abdel-Sayed et al. 2014a). Thus a strong reduction in the ability of cartilage to dissipate energy in early-stage OA may initiate and/or accelerate a cascade of degeneration.

Shear stresses allowed us to distinguish between healthy load-bearing (HL\(_0\)) and degenerated (OA-2 to -4) cartilage, and between normal (OA-1) and degenerated (OA-3 to -4) cartilage. The peak effective shear modulus allowed us to distinguish between healthy load-bearing (HL\(_0\)) and severely degenerated cartilage, or between normal (OA-1) cartilage and severely degenerated cartilage, but only at larger strains for both. The significant changes that in mechanics we report with progressing degeneration are consistent at all magnitudes of applied strain, but are slightly more pronounced at larger strains.

### 5.4.5 Structural integrity

All of our reported mechanical measures decrease 30-40\% when the zonal architecture of cartilage becomes compromised. However, all specimens obtained from TKAs (OA-1 to -4) showed signs of remodeling in the SZ, i.e. a PLM score \(<5\). Buckley et al. (2013) showed that energy dissipation is highest near the transition from SZ to MZ. Thus, the \(\sim 50\%\) decrease in energy dissipation that we report between healthy load-bearing (HL\(_0\)) and normal (OA-1) cartilage from TKAs may originate from minute changes in the zonal architecture near the articular surface.

Desrochers et al. (2012) found, using atomic force microscopy, that the time-dependent mechanics of cartilage under cyclic compression are sensitive to changes associated with
early-stage OA, and they concluded that changes within the collagen network have a greater effect than changes in the composition. We showed the same effects by macro-scale measurements, thus highlighting the potential for clinical application. Current instruments for arthroscopic indentation lack sensitivity to detect early-stage OA (Brommer et al. 2006, Kiviranta et al. 2008). If such instruments could be adapted to measure both the loading and unloading response, a measure of energy dissipation may realize this goal.

5.4.6 Collagen and proteoglycan content, thickness, and age

The strong reduction (~80%) in strain-energy dissipation density we found when comparing normal (OA-1) with severely degenerated tissue (OA-4) can be (partially) attributed to the reduction in GAG content (~33%) typically associated with early-stage OA (Buckwalter et al. 2005, Stolz et al. 2010). However, loss of GAG alone cannot explain the 50% reduction we found when comparing normal cartilage (with both GAG and collagen content similar to those in healthy cartilage) to healthy young cartilage. Initial structural remodeling, and thus a reduction in the zonal integrity reflected in the reduction in PLM-CO scores, may account for the remaining difference.

We found a weak to moderate correlation of all our mechanical measures, except $E_{DI}$ at strain levels 5 and 10%, with GAG content. The strong negative fixed charge of GAG molecules attracts fluid to increase the osmotic pressure within the tissue and pre-tension the collagen network. Thus both the network of collagen and the corresponding bulk tissue response appears stiffer (Nguyen and Levenston 2012). We did not find correlations between any of our mechanical measures and total collagen content, and collagen content did not significantly change with progressing degeneration. This result emphasizes that collagen structure and the zonal architecture play greater roles in cartilage mechanics than does collagen content (Desrochers et al. 2012).

We found no correlations between any of our mechanical measures, nor the OARSI grades, and the thicknesses of our specimens. This result confirms that specimen thickness is a poor
predictor for cartilage health, cf. Guermazi et al. (2011). N.B. specimen thickness can still be used to observe the progression of OA in longitudinal studies if a healthy baseline is established (Huang et al. 2013). We did find that the reduction in specimen thickness correlated with age.

We did not find correlations between any of our mechanical measures and age. Thus, OA-associated degeneration has a greater effect than age on the mechanical behavior of cartilage, cf. Temple et al. (2007).

5.4.7 Limitations and outlook

We included only tissue from lateral femoral condyles although degeneration generally presents first in the medial femoral condyle (Muehleman et al. 1997). However, the mechanical properties of cartilage from the medial and lateral condyles are not significantly different (Deneweth et al. 2015, Santos et al. 2017), thus our findings should apply to both lateral and medial femoral condyles.

Our mechanical results do not represent in-vivo loading rates, where healthy cartilage can generate substantial hydrostatic interstitial fluid pressure. However, our results allow us to decouple the mechanical responses (solid vs. fluid) and to investigate changes in the bulk properties of the solid constituents, thus identifying changes in the solid matrix that may be masked by alterations of the permeability (Workman et al. 2017). Cartilage stiffness, in both tension and compression, is rate dependent but reaches a plateau if the rate of applied deformation is sufficiently slow (Huang et al. 2001, 2003). We found that rate to be 75 μm/min for healthy control cartilage under simple shear, cf. Maier et al. (2017).

We found that the progression of OA significantly changes the mechanical responses of articular cartilage undergoing large-strain shear deformations. Our results suggest that a reduction in energy dissipation can be detected by bulk-tissue testing, and that this reduction precedes visible signs of degeneration. This significant reduction is associated with remodeling of the collagen network and with alterations in the zonal architecture, rather
Table A3: Detailed summary relating each mechanical test (applied strain magnitude) to the patient number and OARSI grade used for statistical analyses. A decreasing number of tests across one row indicates that specimen(s) failed mechanically. The total number of tests shown at the bottom is the number of data available for statistical analyses at each strain magnitude.

than with changes in cartilage composition. Our results highlight the potential of energy dissipation, as opposed to stress- or stiffness-based measures, as a marker to diagnose early-stage OA.

### Appendix P2

We provide a detailed summary relating each mechanical test (applied strain magnitude) to the patient number and OARSI grade used for statistical analyses in Table A3.

We report the quantitative data for shear strain-energy dissipation $E_{DI}$, peak-to-peak shear
Evolving Large-Strain Shear Responses of Progressively OA Human Cartilage

\[ \tau_{PP}, \text{ and peak effective shear modulus } G_{PE} \text{ in Table A4, and percent and absolute differences of these in Tables A5 to A7.} \]

<table>
<thead>
<tr>
<th>( \gamma ) (%)</th>
<th>OARSI Grade</th>
<th>PLM-CO Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>( E_{DI} ) (ml/mm(^3))</td>
<td>0.9143</td>
<td>0.7188</td>
</tr>
<tr>
<td>( \tau_{PP} ) (kPa)</td>
<td>85.24</td>
<td>60.45</td>
</tr>
<tr>
<td>( G_{PE} ) (kPa)</td>
<td>894.0</td>
<td>701.0</td>
</tr>
<tr>
<td>( E_{DI} ) (ml/mm(^3))</td>
<td>194.7</td>
<td>143.8</td>
</tr>
<tr>
<td>( \tau_{PP} ) (kPa)</td>
<td>0.9143</td>
<td>0.7188</td>
</tr>
<tr>
<td>( G_{PE} ) (kPa)</td>
<td>894.0</td>
<td>701.0</td>
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<tr>
<td>( E_{DI} ) (ml/mm(^3))</td>
<td>194.7</td>
<td>143.8</td>
</tr>
<tr>
<td>( \tau_{PP} ) (kPa)</td>
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</tr>
<tr>
<td>( G_{PE} ) (kPa)</td>
<td>894.0</td>
<td>701.0</td>
</tr>
</tbody>
</table>

Table A4: Shear strain-energy dissipation densities \( E_{DI} \), peak-to-peak shear stresses \( \tau_{PP} \), and peak effective shear moduli \( G_{PE} \) given as median and interquartile range \([Q_1,Q_3]\) at all magnitudes of applied strain across all donors and binned by OARSI grades (OA-1 to -4) and PLM-CO scores (\( \geq 3 \) or \(< 3 \)).
Table A5: Relative and absolute differences in shear strain-energy dissipation density ($E_{DI}$) of normal cartilage from TKAs and healthy controls (rows) versus cartilage with progressing OA (columns). OA-1 to -4 denotes the corresponding OARSI grade, HL$_0$ and HN denote healthy controls from load-bearing (under 0$^\circ$ knee flexion) and non-load-bearing regions, respectively. The percent difference is calculated as $\frac{\Delta E_{DI}}{\text{median}(E_{DI},\text{ref})} \times 100$. CI denotes the 95% confidence interval.
Table A6: Relative and absolute differences in peak-to-peak shear stresses $\tau_{pp}$ of normal cartilage from TKAs and healthy controls (rows) versus cartilage with progressing OA (columns). OA-1 to -4 denotes the corresponding OARSI grade, HL₀ and HN denote healthy controls from load-bearing (under 0° knee flexion) and non-load-bearing regions, respectively. The percent difference is calculated as $\Delta \tau_{pp} / median(\tau_{pp,ref}) \times 100$. CI denotes the 95% confidence interval.
Table A7: Relative and absolute differences in peak-effective shear moduli $G_{PE}$ of normal cartilage from TKAs and healthy controls (rows) versus cartilage with progressing OA (columns). OA-1 to -4 denotes the corresponding OARSI grade, HL₀ and HN denote healthy controls from load-bearing (under 0° knee flexion) and non-load-bearing regions, respectively. The percent difference is calculated as $\frac{\Delta G_{PE}}{\text{median}(G_{PE,\text{ref}})} \times 100$. CI denotes the 95% confidence interval.
6 Alterations in the Shear Strain Pattern in early OA

6.1 Introduction

In osteoarthritis (OA) the degenerated cartilage is eventually unable to withstand normal (daily) intra-tissue mechanical loads, and begins a sustained degradation of the ECM Sandell and Aigner (2001), Loeser et al. (2012). Currently no treatment exists to fully restore damaged or degenerated cartilage (Correa and Lietman 2017). Tools to detect/monitor preclinical degeneration of cartilage may facilitate the development and validation of treatments, allowing intervention before cartilage degenerates beyond repair (Casula et al. 2016). Methods to track OA progression based on histological images, e.g. OARSI scoring (Pritzker et al. 2006) or adapted Mankin grading (Mankin et al. 1971), are sensitive to early-stage OA but require tissue explants generally unavailable in a clinical setting. Methods to quantify degeneration of cartilage used in clinical practice rely on arthroscopic evaluation, e.g. Outerbridge (Outerbridge 1961), or magnetic resonance imaging (MRI), e.g. protocol by International Cartilage Repair Society (ICRS) (Brittberg and Winalski 2003), but are currently not sensitive enough to detect the onset of degeneration (i.e. early stage).

Improved imaging techniques based on MRI have the potential for accurate, non-invasive assessment of cartilage health, particularly beyond the articular surface. A challenge remains to identify sensitive and reliable MRI markers associated with early OA. MacKay
et al. (2018) recently highlighted the potential of both $T_2$ and $T_{1\rho}$ relaxometry, with the latter showing better predictive power, to distinguish between cartilage affected by OA and healthy controls. The authors used the Kellgren-Lawrence Grade to classify progression of OA and while this radiographic classification system has wide clinical applications, it is not sensitive to early OA (Kohn et al. 2016). Casula et al. (2016) proposed that the dGEMRIC index, a measure for the proteoglycan (PG) density, may map to degenerated cartilage presenting a macroscopically healthy appearance (ICRS grade 0).

OA-induced degeneration also affects collagen fiber orientation across the cartilage surface, as well as through the thickness. Remodelling of the collagen network in cartilage typically initiates with fibrillation of the superficial zone (SZ) followed by lesions (Goldring and Goldring 2010). Desrochers et al. (2012) found that minute changes in the collagen structure precede other changes (e.g. PG loss in particular) and cause alterations in the mechanical responses of cartilage at the onset of OA. Utilizing small angle X-ray scattering Moger et al. (2007) reported changes in zonal thickness, fiber realignment, and loss of organization in the vicinity of cartilage lesions. Changoor et al. (2011a) confirmed these trends using histological slides observed with polarized-light microscopy (PLM).

Intra-tissue mechanics may thus provide more sensitive image-based biomarkers for degeneration of cartilage. Griebel et al. (2014) found that changes in shear strain magnitude, measured by dualMRI, showed a stronger correlation with OA severity than $T_{1\rho}$ relaxometry. These imaging methods highlight the potential of detecting the onset of OA by leveraging changes in local mechanics caused by OA-induced remodeling of the collagen structure.

In this direction, studies applying digital image correlation (DIC) provided information to better understand the local mechanobiological environment within articular cartilage under compression (Gao et al. 2015, Lai and Levenston 2010a) or shear (Buckley et al. 2008, Motavalli et al. 2013, Wong et al. 2008a). The average bulk shear modulus of healthy bovine cartilage varies from 0.1 to 0.31 MPa Zhu et al. (1986, 1993), Soltz and Ateshian...
Alterations in the Shear Strain Pattern in early OA

(2000), Kurz et al. (2001) under infinitesimal strain. However, the modulus varies by up to two orders of magnitude through the thickness of healthy cartilage, with the superficial zone (SZ) a distinctly soft region with reduced collagen density near the articular surface (Buckley et al. 2008, Silverberg et al. 2013, 2014).

During normal movement cartilage undergoes large deformations (greater than 20% (Liu et al. 2010, Bingham et al. 2008)) generating combined tensile, compressive, and shear strains (Mansour 2003, Athanasiou et al. 2009). As cartilage undergoes large deformations in vivo, mechanical analyses of cartilage should employ large-strain kinematics and nonlinear mechanics. While studies of mechanical shear have shed significant light on the structure-function relationships in cartilage, most do not include large-strain deformations and human tissues, important both for understanding in-vivo mechanics and for constitutive modeling.

Similarly, very few studies utilized DIC to study cartilage under large deformations. Wong et al. (2008a) investigated the influence of lubrication and degeneration on intra-tissue patterns of shear strain within cartilage in contact with a sliding glass surface. The authors found that local, as well as bulk shear strains, increased with tissue degeneration and friction (lubricant–synovial fluid versus phosphate buffered saline (PBS)). Unfortunately they did not investigate the influence of progressing OA but grouped all of their OA cartilage for subsequent analyses. By gluing the articular surface to their testing device, Motavalli et al. (2013) applied large-strain shears (up to 25%) to bovine articular cartilage. The authors compared immature versus skeletally mature cartilage and found differences in the local shear-strain patterns that corresponded with collagen structure.

In light of the structural changes associated with the progression of OA, we hypothesized that patterns of through-thickness, large-strain shear evolve with early-stage OA. We aimed to determine characteristic and consistent changes in the patterns of shear strain during early-stage OA to 1) gain insight into the progression of OA by understanding changes both in the load bearing proteins within cartilage and the mechanobiological environment
of the cells; 2) gauge the potential of these patterns to serve as image-based biomarkers for detection of early-stage, preclinical OA; and 3) provide high-fidelity, through-thickness data for proposing, fitting, and validating constitutive models.

6.2 Materials and Methods

Using a triaxial shear testing device (Messphysik, Fuerstenfeld, AT) we completed displacement-driven, large-strain shear tests (following a protocol detailed previously (Maier et al. 2017)) on 44 specimens of progressively osteoarthritic human articular cartilage as determined by both OARSI grade (Pritzker et al. 2006) and PLM-CO score (Changoor et al. 2011a). We recorded the through-thickness deformations with a stereo camera system and processed these data using DIC to determine full-thickness patterns of shear strains and relative zonal recruitments.

6.2.1 Preparation of Specimens

We harvested nine lateral femoral condyles \( n = 9, 67.0 \pm 11.9 \text{ years old} \) undergoing total knee arthroplasty (TKA) at Hartford Healthcare Bone & Joint Institute and transported them, submerged in PBS, to our lab within eight hours of extraction. After determining the local split-line directions (SLDs) by pricking the articular surface with a needle dipped in India ink, we extracted pairs of adjacent test specimens from locations across the joint cartilages. From each pair, we used one specimen for mechanical testing (cuboid, \( 3 \times 3 \text{ mm}^2 \) footprint, full thickness), with one through-thickness plane parallel to the local SLD, and the other for histological evaluation. We fixed the latter specimens immediately in 10% neutral buffered formalin, see Section 6.2.2.

For the specimens undergoing mechanical testing we carefully removed all of the underlying trabecular bone and sufficient subchondral bone to create surfaces parallel to the articular surfaces. We left the bone-cartilage interfaces intact to ensure boundary conditions
mimicking the in-situ conditions (Morel and Quinn 2004). We stored these specimens submerged in PBS at $-80^\circ$ prior to testing. On the day of a mechanical test, we thawed and glued a specimen to a loading platen, ensuring a flat surface perpendicular to the stereo camera setup (front surface), and uncontaminated by glue (Fig. 6.1(a,b)). We then airbrushed (CM-C Plus, IWATA, Yokohama, JP) a speckle pattern on the front surface using a tissue marking dye (CDI’s tissue Marking Dyes, Cancer Diagnostics, Durham, USA) diluted with deionized water (approximately 1:1). We let the speckle pattern air dry for 10 minutes, while keeping the other surfaces coated by PBS to prevent tissue dehydration and shrinking (Fig. 6.1(c,d)).

Figure 6.1: Representative specimen glued to loading platen (black): (a) front, (b) side, and (c) back views. In (b) we see the flat surface perpendicular to the stereo camera setup. In (c) we see three sides of the specimen covered with PBS (black arrows) while the speckle pattern dries. In (d) we see the final speckle pattern used for DIC recording, white arrows indicate the bone-cartilage interface. Scale bars equal 1 mm.

6.2.2 Histological Assessments

After decalcification in 0.5 M EDTA, we embedded specimens for histology in paraffin and sectioned them at 6 $\mu$m. To determine overall cartilage health we stained sections with Safranin-O fast green (Novaultra Safranin O stain kit, IHC World, Woodstock, USA) and examined the slides using a light microscope. Two trained observers (FM, DMP) quantified the progression of OA using the OARSI grading method (Pritzker et al. 2006). We considered only tissues up to grade 3.5 (moderate disease, presence of vertical fissures). For statistical analyses we binned specimens into three groups based on their OARSI grades:
normal cartilage (grade $< 2$, group OA-1), mild OA ($2 \geq \text{grade} < 3$, group OA-2), and moderate OA (grade $\geq 3$, group OA-3). To assess the presence and integrity of the through-thickness zonal architecture we stained sections with Picosirius red (Novaultra Sirius red stain kit, IHC World) and examined the slides under polarized light. We first oriented the analyzer at $45^\circ$ to the articular surface and then oriented the polarizer to achieve maximum extinction. Two trained observers (FM, LM) quantified the integrity of the zonal architecture using the PLM-CO score (Changoor et al. 2011a). This score uses an ordinal scale, ranging from score 0 (completely disorganized architecture) to score 5 (normal, healthy zonal architecture). For statistical analyses we binned specimens into three groups based on their PLM-CO scores: normal collagen structure (score $\geq 4$), mild disruption (score 3), and moderate disruption (score $\leq 2$).

### 6.2.3 Quantification of Constituents

To quantify the mass fractions of the constituents, we deparaffinized the embedded specimens, removed any subchondral bone, and measured their dry weights. We then rehydrated the specimens with a decreasing alcohol series to PBS (van Wijk et al. 2012), measured their wet weights, and cut a 10 mg pieces for analyses. We completed digestion and analyses using a Glycosaminoglycan Assay Kit (6022, Chondrex, Redmond, USA) and a Hydroxyproline Assay Kit (6017, Chondrex). We solubilized each 10 mg specimen in 1.25 ml digestive solution (125 $\mu$g/ml Papain; 60224, Chondrex) in PBS at pH 6.3 with 5 mM L-cystein-HCL and 10 mM EDTA-2Na, incubated at 65°C for 36 hours. We then centrifuged this solution at 10,000 rpm for five minutes and transferred the supernatant for analyses with the GAG assay. After determining the GAG concentration, we hydrolyzed 100 $\mu$L of the dissolved tissue in 10 N hydrochloric acid and quantified the hydroxyproline concentration. We multiplied this concentration by 7.4 to get total collagen (following the protocol from Chondrex). We reported all concentrations per wet weight.
6.2.4 Digital Image Correlation During Applied Shear Strains

To apply prescribed shear strains, we used our testing device and protocol as previously described in Maier et al. (2017). Briefly, we performed quasi-static (75 µm/min) cyclic simple shear tests, and recorded images of the deformation in the SLD, i.e. with the front plane aligned with the SLD. We applied a 1% precompression and applied displacements corresponding to ±5, ±10 and ±15% shear strain. At each step in our protocol we allowed specimens to equilibrate, i.e. after gluing (2000 sec), after precompressing (4000 sec), and after increasing the strain magnitude (600 sec). Our cyclic tests included three preconditioning cycles and we recorded images of the fourth loading cycle to calculate patterns of displacement and strain. After completion of the full testing protocol we applied tension to the specimen to verify the integrity of the glued interface. We completed all tests in a bath of PBS at 37°C (±1°C) including antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) and protease inhibitors (P2714, Sigma Aldrich, St. Louis, USA) to avoid tissue degeneration.

To record images for subsequent DIC we used a stereo camera system with two five megapixel cameras (Manta G-505, Allied Vision, Stadtroda, DE). We adjusted the frame rate depending on the applied displacement to record both loading and unloading in approximately 200-250 frames. To ensure accuracy of our measurements we calibrated the camera setup before each test, and after allowing the bath of PBS to reach temperature.

6.2.5 Data Analyses

We used the commercial software Istra4D (V4.4.3.414, Dantec Dynamics, Skovlunde, DK) for image processing and strain calculations. Briefly, to track the deformation we established a grid pattern on the cartilage surface based on the speckle pattern. Our grid spacing of 10-15 pixels (depending on image quality) and pixel size of 3 µm resulted in a data point every 30-45 µm. We applied a local regression filter for the object contour based on
6.2 Materials and Methods

an adaptive spline polynomial algorithm with a $5 \times 5$ kernel size. Applying Istra4D we approximated the displacement field by an analytic function (bi-cubic splines) to obtain 2-D deformation gradients for subsequent strain calculations. We used a grid-reduction factor of two (only direct-neighbor facets influence smoothing) and a smoothness factor of minus one (higher absolute value increases smoothing) for filtering (both manufacturer recommended). We processed our image data through the full thickness of each specimen, from the articular surface to the subchondral bone. In our images the transition from cartilage to bone was clearly visible as a dark line (cf. Fig. 6.1(d)), and this served as a marker for the lower boundary. We exported the full field of 2-D Green-Lagrange tangential shear strain calculated as

$$E_{XY} = \frac{1}{2} \left( \frac{\partial u_X}{\partial Y} + \frac{\partial u_Y}{\partial X} + \frac{\partial u_X}{\partial Y} \frac{\partial u_X}{\partial Y} + \frac{\partial u_Y}{\partial X} \frac{\partial u_Y}{\partial X} \right). \quad (6.1)$$

We performed further processing and analyses using MATLAB (R2017b, The MathWorks, Natick, USA). First, we averaged (mean ± standard deviation) the shear strain values along horizontal lines at each height through the thickness of the specimen to generate a through-thickness strain curve. To avoid edge effects (cf. Wong et al. (2008a)) we excluded approximately 10% of our data on each side of the front area (from the edges inwards). We then grouped those curves by either OARSI grade or PLM-CO score (Section 6.2.2) and averaged them to obtain master curves representing normal, mildly degenerated, and moderately degenerated cartilage. We also calculated the relative zonal recruitment $R_i$ with $i \in \{ DZ, MZ, SZ \}$, i.e. the average shear strain in a through-thickness zone weighted by its relative thickness and normalized by the applied strain, as

$$R_{DZ} = \frac{t_{DZ} E_{XY,DZ}}{E_{XY,blk}}, \quad R_{MZ} = \frac{t_{MZ} E_{XY,MZ}}{E_{XY,blk}}. \quad (6.2)$$
where \( t_i \) is the relative thickness of the corresponding zone, i.e. 0.3 for the DZ and 0.5 for the MZ (Mow et al. 2005), \( E_{XY,i} \) is the averaged Green-Lagrange shear strain in zone \( i \). We calculated the bulk shear strain as \( E_{XY, blk} = \gamma_{app} / 2 \), with \( \gamma_{app} = u_{X,max} / t \), where \( u_{X,max} \) is the maximum applied displacement causing shearing and \( t \) is the thickness of the cartilage (top loading platen to the subchondral bone) measured from the DIC images. To account for data potentially missing near the articular surface (we considered up to 3% of the total thickness acceptable), we calculated the relative zonal recruitment of the superficial zone \( R_{SZ} \) as

\[
R_{SZ} = 1 - (R_{MZ} + R_{DZ}). \quad (6.3)
\]

In this way the sum of the three zonal recruitments always equals 100% recruitment.

### 6.2.6 Statistical Analyses

We first confirmed, using a Shapiro-Wilk test, that our data was normally distributed. To validate inter-observer agreement in our application of the OARSI and PLM-CO grading methods we calculated the linear-weighted Cohen’s kappa coefficient \( \kappa \). We used an ANOVA analysis followed by a Tukey-Kramer post-hoc test to compare the relative zonal recruitment for each zone (SZ, MZ, and DZ) by OARSI and PLM-CO groups (Section 6.2.2). Finally, we used Pearson’s correlation coefficient to determine the effect of age, specimen thickness, and collagen and GAG content on the relative zonal recruitment. We adjusted the level of significance with the Bonferroni-Holm adjustment to account for multiple comparisons.
6.3 Results

We completed a total of 132 shear tests using 44 cartilage specimens. We successfully imaged the distribution of deformation at three applied strain levels (5, 10, 15%) on most of the 44 specimens. Seven of our specimens showed signs of mechanical failure during testing (one at 10% and six at 15% applied strain), visible as an instant drop in the measured force response, and we excluded these data from subsequent analyses.

6.3.1 Histological Assessments

We show representative images of histological slides stained either with Safranin-O visualized under white light or stained with Picosirius-red and visualized under polarized light in Fig. 6.2. Our OARSI grading resulted in $n_{OA-1} = 17$, $n_{OA-2} = 16$, and $n_{OA-3} = 11$ specimens per binned group. Inter-observer agreement for the unbinned OARSI grades was 0.925 and for the binned OARSI-graded groups was 1.0. Our PLM-CO scoring resulted in $n_{PLM-2} = 18$, $n_{PLM-3} = 12$, $n_{PLM-4} = 10$, and $n_{PLM-5} = 4$ specimens per binned group. For further statistical analyses we binned specimens with scores 4 and 5 into one group. Inter-observer agreement for the unbinned PLM score was 0.735 and for the binned PLM score 0.766.

6.3.2 Quantification of Constituents

We found similar trends as described in Section 5.3.2.

6.3.3 Through-Thickness Patterns of Shear Strain

We observed three general shapes for the curves of averaged through-thickness Green-Lagrange shear strains consistent over all applied strain magnitudes, as shown in Fig. 6.3. In all specimens we found the highest shear strains (by magnitude) at or near the articular surface. Normal tissue (OA-1) also showed a distinct relative peak in shear strain near
Figure 6.2: Representative images of histological slides stained with Safranin-O fast green and visualized under white light (a)–(c) for assessing the severity of osteoarthritis using the OARSI grading method: (a) OARSI grade 1 (OA-1)—intact surface and proteoglycan (PG) content, (b) OARSI grade 2 (OA-2)—increased surface roughness and loss of PG, (c) OARSI grade 3 (OA-3)—vertical fissures and greater loss of PG. Red indicates PG. Representative images of histological slides stained with Picosirius red and visualized under polarized light (d)–(g) for assessing the microstructural arrangement of collagen using the PLM-CO scoring method: (d) PLM-CO score 5: normal zonal architecture, (e) PLM-CO score 4: proportional but laterally heterogeneous zones, (f) PLM-CO score 3: present but disproportional zones, and (g) PLM-CO score 2: intact deep zone but missing others. Bright yellow indicates areas with fibers aligned parallel to the articular surface, black indicates randomly isotropic alignment, and bright blue-green indicates areas with fibers aligned perpendicular to the surface. Bars indicate 500 \( \mu m \)
Figure 6.3: Representative through-thickness patterns of 2-D Green-Lagrange shear strain at applied bulk shear strains $\gamma = 5\%, 10\%, 15\%$ (columns 1, 2, 3) and corresponding averaged through-thickness shear strain response (column 4) with progressing osteoarthritis, i.e. increasing OARSI grade 1, 2, 3 (rows 1, 2, 3).
the transition from the MZ to the DZ, near or at the advancing cartilage-bone interface (Fig. 6.3(d)). With advancing OA (mild degeneration, OA-2) the through-thickness shear strain became more homogeneous (Fig. 6.3(h)). Finally, with further advancing OA (moderate degeneration, OA-3) the shear strain became more heterogeneous with most of the deformation focused near the articular surface (Fig. 6.3(l)).

When we averaged the shear-strain response of all specimens binned by OARSI grade we observed the same three general curves (Fig. 6.4), however the standard-deviation of the mean of the averaged curves does not show a clear distinction between all curves in all segments.

When we plotted the relative zonal recruitment, binned by OARSI grades Fig. 6.5) and PLM scores (Fig. 6.6) we observed that the only consistently significant difference in zonal recruitment during the progression of OA is in the DZ. When grouped by OARSI grade, at 10% applied shear strain zonal recruitment in the SZ significantly increased with progressing OA, and zonal recruitment in the MZ and DZ increased in mild versus moderate OA. When grouped by PLM score a consistent distinction in the zonal recruitment in the DZ became evident. Zonal recruitment in the DZ was significantly larger with a healthy or near-healthy organization of collagen (PLM score ≥ 4) than any other groups. There was no significant difference in zonal recruitment for the MZ or SZ across PLM-CO scores.

### 6.3.4 Statistical Analyses

We summarize the correlations of zonal recruitment with collagen content, age and thickness in Table 6.1. Both bulk collagen content and age correlate significantly with increased zonal recruitment in the SZ and correspondingly reduced zonal recruitment in the DZ. Specimen thickness only correlates significantly at 15% applied strain where zonal recruitment is greater in the SZ and correspondingly reduced in the DZ. We found no significant correlations among zonal recruitments and bulk GAG content. All correlations were relatively weak but significant.
Figure 6.4: Through-thickness patterns of 2-D Green-Lagrange shear strain averaged by grouped OARSI grades at applied bulk shear strains $\gamma = 5\%, 10\%, 15\%$, (a), (b), and (c) respectively.
Figure 6.5: Relative zonal recruitment binned by grouped OARSI grades at applied bulk shear strains $\gamma = 5\%, 10\%, 15\%$, (a), (b), and (c) respectively. Black bars indicate significant differences ($p < 0.05$) between OARSI grades per zone.
Figure 6.6: Relative zonal recruitment binned by grouped PLM scores at applied bulk shear strains $\gamma = 5\%, 10\%, 15\%$, (a), (b), and (c) respectively. Black bars indicate significant differences ($p < 0.05$) between PLM scores per zone.
Table 6.1: Pearson correlation coefficient $r$ and corresponding level of significance $p$ for correlation of relative zonal recruitment with collagen content, age, and thickness at applied bulk shear strains $\gamma = 5\%, 10\%, 15\%$. N.B. Only results shown with $p < 0.05$. 

<table>
<thead>
<tr>
<th>$\gamma$ (%)</th>
<th>Zone</th>
<th>Collagen</th>
<th>Age</th>
<th>Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>SZ</td>
<td>$0.4007$</td>
<td>$0.3396$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$0.0085$</td>
<td>$0.0278$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MZ</td>
<td>$-0.3816$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$0.0127$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DZ</td>
<td>$-0.3120$</td>
<td>$-0.5033$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$0.0442$</td>
<td>$0.0007$</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>SZ</td>
<td>$0.4977$</td>
<td>$0.3536$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$0.0009$</td>
<td>$0.0233$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MZ</td>
<td>$-0.4428$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$0.0037$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DZ</td>
<td>$-0.4204$</td>
<td>$-0.6068$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$0.0062$</td>
<td>$&lt;0.0001$</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>SZ</td>
<td>$0.5712$</td>
<td>$0.4804$</td>
<td>$-0.3410$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$0.0004$</td>
<td>$0.0040$</td>
<td>$0.0484$</td>
</tr>
<tr>
<td></td>
<td>MZ</td>
<td>$-0.5068$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$0.0022$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DZ</td>
<td>$-0.4297$</td>
<td>$-0.7464$</td>
<td>$0.4897$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$0.0112$</td>
<td>$&lt;0.0001$</td>
<td>$0.0033$</td>
</tr>
</tbody>
</table>
6.4 Discussion

We successfully imaged the through-thickness patterns of deformation and calculated patterns of shear strain in articular cartilage in the early stages of OA and under large deformations. We found that progression of early-stage OA presents characteristic through-thickness patterns of shear strain for normal cartilage, and mild and moderately degenerated cartilage. We also found that progression of early-stage OA and loss of the through-thickness zonal architecture of collagen fibers, measured as OARSI grade and PLM-CO score respectively, only significantly alter zonal recruitment in the DZ of human articular cartilage during shear deformations.

6.4.1 Histological Assessments

Semi-quantitative, histology-based methods for assessing cartilage degeneration have inherent drawbacks, e.g. these discrete categories likely can not fully capture the dynamic process of disease progression, spanning over years, in its full complexity. We quantified the OARSI grade rather then the complete OARSI score (Pritzker et al. 2006), to estimate local degeneration. Despite the good to excellent inter-observer agreement reported here, application of OARSI grading and PLM scoring is still subjective and automated evaluation tools may reduce potential bias Zhang et al. (2015).

We attempted to utilize the histological images from slides stained with Picosirius red to extract specimen-specific zonal thicknesses for each specimen as a mask to calculate zonal recruitments. Unfortunately not all specimens showed three distinct through-thickness zones, despite the early stages of degeneration, indicating that tissue remodeling precedes other signs of disease progression. Thus, we selected a generic mask (0.2/0.5/0.3 for SZ/MZ/DZ) based on Mow et al. (2005). Additionally, this idealized mask could serve in the future as a clinal diagnostic marker since the real in-vivo zonal architecture is generally not obtainable.
6.4.2 Quantification of Constituents

We found similar trends as described in Section 5.3.2.

6.4.3 Through-Thickness Patterns of Shear Strain

We found three general qualitative through-thickness patterns of shear strain depending on the stage of degeneration of the specimens (cf. Fig. 6.4). Across all stages of degeneration (normal, mild, moderate) we found the highest strains, by magnitude and relative contribution, in the SZ. However, only a few specimens showed a distinct increase in shear strain in the transition between SZ and MZ, first reported by Buckley et al. (2008) for healthy neonatal bovine tissue, and subsequently confirmed for human tissue (Buckley et al. 2010). Most of our specimens showed through-thickness patterns of shear strain at the SZ comparable to Wong et al. (2008a). They tested human articular cartilage in health and disease and found highest shear strains at the articular surface and without a distinct increase in shear strain in the transition between SZ and MZ.

We are the first to report a region of increased shear strains in normal cartilage in the transition from MZ to DZ, i.e. near the cartilage-bone interface (cf. Fig. 6.3(a-d)). Lai and Levenston (2010a) performed unconfined compression tests on cartilage explants and showed a similar increase in shear strain near the cartilage-bone interface. However, this was much smaller by magnitude than shear strains near the surface and thus they did not comment on it further.

Our relative zonal recruitment provides information on the contribution of each through-thickness zone to the total deformation of a specimen. Interestingly, the relative zonal recruitment of the DZ reduces with progressing OA (OARSI grade) and microstructural remodeling (PLM-CO score), while the MZ and SZ are not consistently (at all applied strain magnitudes) significantly affected. The DZ of all specimens appeared intact (PLM-CO > 1) while we observed degeneration of the zones above, i.e. changes in the thickness ratios
of SZ and MZ, as well as complete remodeling of the SZ. Thus, while OA initiates at the articular surface (Hollander et al. 1995), the patterns of shear strain in the DZ and, as a consequence, the mechanobiological environment of the chondrocytes within, are affected in the early stages of both OA (OARSI grade > 2.5) and collagen-network remodeling (PLM-CO score ≤ 4). The mechanosensitivity of chondrocytes (Grodzinsky et al. 2000) suggests that the alteration in environment may stimulate changes in matrix synthesis, potentially initiating or accelerating a degenerative cascade. The formation and re-absorption of calcified cartilage also corresponds to changes in the biomechanical environment (O’Connor 2009, Schultz et al. 2015), thus favoring or potentially initiating advancement of the calcification front, i.e. tidemark duplication, associated with OA (Mansfield and Peter Winlove 2012) and often accompanied by collagen fibrillation at the articular surface (Revell et al. 1990).

Grouping the zonal recruitments by PLM-CO score showed that strains in the DZ are higher when the zones above are intact, thus it appears that softening of the SZ and MZ, rather than stiffening of the DZ, causes the reduction in shear strain. However, improved imaging modalities capable of accurately imaging the tidemark, e.g. (Mansfield and Peter Winlove 2012), and through-thickness indentation tests (Hargrave-Thomas et al. 2015) could further clarify this point.

Advantageously, our experiments have very little out-of-plane deformation (confirmed by our stereo camera setup) and our applied speckle pattern is not heterogeneously compressed, both effects aiding the quality of our DIC. The quasi-static nature of our experiments does not represent deformation rates in vivo, where healthy cartilage can generate substantial interstitial fluid pressure. However, our experiments do allow us to measure patterns of deformation unaffected by inter-tissue fluid flow and to investigate local deformations of the solid constituents, thus identifying changes in the solid matrix that may be masked by alterations in bulk permeability (Workman et al. 2017).
6.4.4 Statistical Analyses

Interestingly, bulk collagen content correlated with local tissue deformation. In terms of zonal recruitment, there is a shift from DZ to SZ (the SZ is progressively recruited more) with age and, at large strains only, with specimen thickness. These correlations, while statistically significant, are relatively weak.

6.4.5 Limitations and Outlook

Large shear strains, applied as simple shears may not mimic the deformation in vivo, dominated by compressive loading. However, despite predominately external compression to cartilage in vivo, shear strains within the tissue tend to exceed compressive strains (Chan et al. 2016). Additionally, Elsaid et al. (2005) showed that lubrication of the contact surfaces reduces in OA, and thus shear deformations caused by higher friction may play an increasing role in OA.

We included only cartilage from the lateral femoral condyle, and did not include any from the medial condyle although degeneration generally occurs there first Muehleman et al. (1997). However, mechanical properties of lateral versus medial condyle appear indistinguishable Deneweth et al. (2015), thus the increased incidence of OA seems to be a consequence of differences in loading rather than tissue mechanics.

In this study specimens with a normal appearance still originate from knees affected by OA, and thus we do not have a fully healthy group as a control. Our normal group may still present compromised load-bearing capabilities despite near-healthy appearances Desrochers et al. (2012), Casula et al. (2016). In OA the synovial fluid contains more matrix degrading proteins Roach et al. (2005) and lubricates the cartilage interfaces less Elsaid et al. (2005), thus affecting the entire joint. Furthermore, due to technical challenges we were not able to test a sufficient number of cartilage explants presenting severe signs of degeneration (OARSI grade > 4). Never-the-less our main aim was to study early-stage
OA to assess/detect preclinical OA Hadjab et al. (2017), thus the focus of our study was achieved.
The following sections list a variety of ideas on how to either strengthen the previous studies or provide ideas for follow up studies to address some of the open questions.

7.1 Mechanical Testing

Pulling on the glued specimen after the last displacement cycle enables us to detect failure of the gluing interface. This allows us to distinguish between mechanical failure of the specimen or the gluing interface, where the later appears more frequent. Mechanical failure of the specimen occurs less drastic, i.e. there is rarely a sharp drop in the force response but rather a gradual softening. An additional test cycle at the smallest tested displacement magnitude after the last applied test could help to determine if this softening relates to actual tissue damage (plastic deformation) or is rather preconditioning-like (reversible).

In addition to calculating the energy dissipation, i.e. area within the stress-strain curve, the total energy from the deformation, i.e. area underneath the loading curve, can be incorporated as well. Calculating a ratio of dissipated to total energy might allow for better comparisons between specimen affected by degeneration.

The potential role of energy dissipation as a sensitive predictor for early stage OA must be further investigated. To clarify if this observation is unique to cartilage under shear a
quick follow up study conducting compression tests on degenerated tissue explants can be performed. A more detailed analysis would incorporate indentation tests on whole, extracted joints or large segment, to test the effect of in-situ boundary conditions. A detailed analysis of the rate dependence can shed light into the mechanisms behind the reduction of the tissue’s energy dissipation. To the extend of our current knowledge, it appears that remodeling and degeneration of the solid matrix mainly contributes to this alteration. As OA effects a multitude material properties certain testing conditions might mask our finding, while others amplify them. To serve as a in-vivo diagnostic tool multiple factors, such as reliability, and sensitivity, but also ease-of-use and acquisition time, must be considered. AFM could be utilized to further reveal the underlying mechanisms responsible for the reduction in DZ strain magnitude and contribution. The nondestructive nature of those test would allow to perform them prior to shear testing. Allowing us to obtain a detailed stiffness and deformation map of the same surface. This enables us to clarify the contribution of SZ and MZ softening and DZ stiffening on the observed behavior.

7.2 Imaging

Improved image modalities can be incorporated to further resolve the through-thickness distribution and orientation of the constituents. A microscope equipped with circularly polarized light would improve the quantification of the collagen orientation from histological slides (c.f. Motavalli et al. (2014)). This setup would allow to analyze the slide without rotating it, thus aiding with image alignment. Further, it would be possible to analyze unstained slides, allowing for immediate results rather than waiting for the fixation and decalcification.

By measuring infrared absorbance via Fourier transform infrared imaging the through-thickness density distribution of collagen and PG can be determined (Silverberg et al. 2014). However, necessary tissue sectioning is already a disruptive mechanism, poten-
tially effecting the constituents (Hunziker et al. 2014, Kansu et al. 2017). Utilizing contrast agents allows to bypass this shortcoming and measure the PG distribution over a volume (Silvast et al. 2013). Agents like sodium iodide accumulate in the vicinity of PG molecules and increase x-ray absorbance. This allows high resolution imaging with contrast enhanced computer tomography (CECT).

Optical coherence tomography (OCT) allows to visualize the microstructure of tissue explants or in-situ configurations. Detection of backscattered low coherence near infrared light allows for a micrometer resolution (Thiboutot et al. 2018). Further, OCT shows good inter- and intra-observer agreement as a diagnostic tool to detect early stage OA (Pailhé et al. 2018, Goodwin et al. 2018). Utilizing this method would enable us to assess the integrity of a specimen prior to shear testing and to potentially detect induced damage once the test is completed. Alternatively, DT-MRI measurements would allow to determine collagen fiber distribution and density over an entire specimen volume (Pierce et al. 2010). However, associated with a higher cost and acquisition time.

Further, our new laser scanning microscope (LSM510, Zeiss), equipped with a tunable infrared light source allows us to detect the SHG signal and thus enables label-free imaging of collagen. Imaging the articular surface and quantifying the defects might allow us to stage disease progression of the specimen prior to shear testing Kumar et al. (2015), Kaleem et al. (2017). This would remove potential errors that arises from utilizing adjacent tissue pieces. Additionally, imaging the tidemark of the specimen would allow precise measurement of the thickness of cartilage and calcified cartilage (Mansfield and Peter Winlove 2012). Equipping our setup with a 5 or 10x objective with a low numeric temperature would be a cheap way to reduce scan time and allow for cost- and time-efficient measurements.

High resolution µCT measurements, or as a low-cost alternative X-ray absorption measurements of the subchondral bone would allow to quantify the bone density. Subchondral bone and calcified cartilage remodel in response to disease and alteration in the mechanobi-
oligical environment Haghighat et al. (2011), Pragnre et al. (2018). If potential changes correlate with mechanical alterations in the above cartilage they could be utilized as an easily-accessible in-vivo biomarker.

7.3 Biology

Animal models might be a necessity to further validate the sensitivity of energy dissipation to early stage degeneration. OA models such as anterior cruciate ligament transection (ACLT) (Schultz et al. 2015, Pragnre et al. 2018) or joint immobilization (O’Connor 2009) would allow for controlled disease progression.

A validation and verification of the method used in Chapter 5 to determine total GAG and Collagen content will strengthen the proposed results. Therefore fresh, frozen, and paraffin embedded tissue specimens from adjacent locations have to be extracted and analyzed with the described Chondrex assay kits (or similar alternatives). As a general guideline, it is recommended to use fresh or frozen specimen.

Utilizing a immunohistochemical detection kit, specific to collagen type II, would further strengthen the biochemical analysis. With progressing disease, type I collagen can be found in cartilage, which can not be resolved when only total collagen (hydroxyproline) is detected. However, this would require tissues with intact epitopes for the antibodies, thus only allows for fresh or frozen specimens.

With advancing age, advanced glycation end products (AGEs) accumulate in cartilage. Those molecules form cross-links between collagen fibrils and alter the mechanical response (Pouran et al. 2018). This mechanism increases tissues stiffness and thus would counter the softening generally observed with progressing OA. However, an intact mechanobiological environment can prevent AGEs from accumulating. Histochemical analysis can quantify the extend of increased cross-links and their role in maintaining a healthy tissue.
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