UNIVERSITY OF CALIFORNIA, SAN DIEGO

# AGE- AND SITE- ASSOCIATED BIOMECHANICAL WEAKENING OF HUMAN ARTICULAR CARTILAGE: RELATIONSHIP TO CELLULARITY, WEAR, MATRIX FRAGMENTATION AND THE PROGRESSION TO OSTEOARTHRITIS

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Bioengineering

by

Michele M. Temple

Committee in charge:

Professor Robert L. Sah, Chair
Professor David Amiel
Professor Richard D. Coutts
Professor Jefferey H. Price
Professor Geert W. Schmid-Schonbein

2005

The dissertation of Michele M. Temple is approved, and it is acceptable in quality and form for publication on microfilm:

_____

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2005

# TABLE OF CONTENTS

# LIST OF FIGURES AND TABLES

# ACKNOWLEDGMENTS

This work would not have been possible without the support of many people. Dr. Robert Sah, who introduced me to Bioengineering research, instilled in me a sense of scientific curiousity and, through his enthusiasm for cartilage research, encouraged me to perservere through rough patches. I would like to thank him for the time and support he gave in so many ways.

I would like to acknowledge support by the National Science Foundation, the Arthritis Foundation, and the National Institutes of Health.

The dissertation author thanks the coauthors for their contributions. The coauthors of Chapter 2 are Won Bae, Michael Chen, David Amiel, and Richard Coutts. For Chapter 3, the coauthors are Won Bae, Michael Chen, William Bugbee, David Amiel, and Richard Coutts. For Chapter 4, the coauthors are Won Bae, Kyle Jadin, Michael Chen, and Jeffrey Price, and for Chapter 5, the coauthors are Yang Xue and Michael Chen. Their assistance with these works was greatly appreciated.

Several collaborators have provided valuable scientific insight, samples, equipment, and time to these projects. I would like to thank my thesis committee, Drs. David Amiel, Richard Coutts, Jeffrey Price, and Geert Schmid-Schonbein, for their time and guidance in all of these projects. Dr. William Bugbee and visiting orthopaedic surgeons, Drs. Kenji Yamada, Kenji Kobayashi, Tsuyoshi Watanabe, and Hirohito Mitsuyama I thank for their time and skill at harvesting samples for these studies. Karen Bowden and Robert Healey I thank for their time spent on histological preparation and histomorphometric analysis.

To the Bioengineering Department at UCSD, including the countless number of people in the administrative and graduate student affairs office, I would like to offer thanks. Irene Jacobo and many of the Bioengineering department administrative staff worked quietly behind the scenes, allowing me the comfort of not having to worry about what deadlines were approaching, making sure I was on the right track with my education, and completing all the right paperwork.

The staff of the Cartilage Tissue Engineering (CTE) Lab have provided much support over the years. Lisa Lottman-Lin and Michael Voegtline kept the lab running as smoothly as possible and provided some semblance of order in a very large lab. I would also like to acknowledge Dr. Albert Chen for his support of the mechanical testing systems, Dr. Won Bae for his work on many projects in this thesis and his valuable statistics support, Barb Schumacher for imparting her knowledge of microscopy and cellular biology, Van Wong for his computer assistance, and Carrie Wirt and Leo Schumacher for their administrative assistance.

CTE graduate students, past and present, have provided much advice and support over the years. I'd like to thank Dr. Doug Chang, my graduate student mentor, for conveying to me an enthusiasm for research and Drs. Mike DiMicco, Kelvin Li, Melissa Curtis, and Chad Lewis for advice and support in the lab when needed. Kyle Jadin for his endless amount of Matlab support, Megan Blewis, Eun Hee Han, Jennifer Hwang, Steven Moseley, Gayle Nugent, and Greg Williams for their time in testing and analysis of samples and proofreading manuscripts, and the many, many others for their lively conversation and gossip, I thank you. It seems like the CTE lab was never a dull place.

There are also many undergraduate students I had the privilege to work with. For me, they were the "work-horses" and, since I was one once, I would like to acknowledge them for their willingness to do the grunt-work and complain only a little. In particular, I

would like to thank Yang Xu, Omar Bissar, Gloria Ao Ieong, Kevin Hsu, Edward Lin, and Scott Tcheng for their laboratory assistance. Most importantly, I would like to thank Mike Chen, who worked with me the longest, for the immeasureable amount of time and support he provided to me and hours of conversation to go with it. I hope that, despite all of my sarcasm, I imparted some knowledge and curiosity about scientific research to them.

Finally, and above all, I would like to thank my family for their unconditional love and support. It is, partly, their encouragement and advice that kept me going through these graduate school years. Finally after the many years of "When are you going to graduate?" I can say that I am finished, and no, I'm really not going to be a professional student for the rest of my life, although I know I'll never stop learning. To my parents, much thanks for always believing in me, and to my husband, Van Wong, thank you for all of your help, whether offered or just taken, and constancy always.

# VITAE

| | |
|---|---|
| 1999 | B.S., Bioengineering<br>University of California, San Diego, La Jolla, California |
| 1999-2004 | Graduate Student Researcher<br>Cartilage Tissue Engineering Laboratory<br>University of California, San Diego, La Jolla, California |
| 2002 | M.S., Bioengineering<br>University of California, San Diego, La Jolla, California |
| 2005 | Ph.D., Bioengineering<br>University of California, San Diego, La Jolla, California |

### Book Chapters

Sah RL, Klein TJ, Schmidt TA, Albrecht DR, Bae WC, Nugent GE, McGowan KB, Temple MM, Jadin KD, Schumacher BL, Chen AC, Sandy JD: Articular cartilage repair, regeneration, and replacement. In: *Arthritis and Allied Conditions. A Textbook of Rheumatology,* ed by WJ Koopman, Lippincott, Williams & Wilkins, Philadelphia, 2005, pp 2277-301.

Chen AC, Klisch SM, Bae WC, Temple MM, McGowan KB, Gratz KR, Schumacher BL, Sah RL: Mechanical characterization of native and tissue-engineered cartilage. In: *Methods in Molecular Medicine: Osteoarthritis: Methods and Protocols*, ed by M Sabatini, F DeCeuninck, and P Pastoureau, Humana Press, Totowa, NJ, 2004, pp 157-90.

### Journal Articles

Nugent GE, Law AW, Wong EG, Temple MM, Bae WC, Chen AC, Kawcak CE, Sah RL: Site- and exercise-related variation in structure and function of cartilage from equine distal metacarpal condyle. *Osteoarthritis Cartilage* 12:826-33, 2004.

Bae WC, Temple MM, Amiel D, Coutts RD, Niederauer GG, Sah RL: Indentation testing of human cartilage: sensitivity to articular surface degeneration. *Arthritis Rheum* 48:3382-94, 2003.

Chen AC, Temple MM, Ng DM, Verzijl N, DeGroot J, TeKoppele JM, Sah RL: Induction of advanced glycation end products and alterations of the tensile properties of articular cartilage. *Arthritis Rheum* 46:3212-7, 2002.

Sonoda M, Harwood FL, Amiel ME, Moriya H, Temple MM, Chang DG, Lottman LM, Sah RL, Amiel D: The effects of hyaluronan on tissue healing following meniscal injury and repair in a rabbit model. *Am J Sports Med* 28:90-7, 2000.

### Selected Abstracts

Sonoda M, Harwood F, Amiel M, Kobayashi K, Moriya H, Temple M, Chang D, Lottman L, Sah R, Amiel D: Characterization of tissue healing following meniscal injury and repair: the effect of hyaluronan treatment. *Trans Orthop Res Soc* 23:887, 1998.

Temple MM, Chang DG, Bae WC, Clark JJ, Gutierrez E, Sonoda M, Kobayashi K, Amiel D, Sah RL: Effect of meniscal repair and hyaluronan injection on cartilage degeneration of the femur and tibia in the rabbit. *Trans Orthop Res Soc* 24:669, 1999.

Temple M, Bae WC, Dounchis JC, Lee MC, Kobayashi K, Coutts RD, Amiel D, Sah RL: Articular cartilage degeneration occurs opposite to lesions of meniscus and cartilage in the rabbit knee. *Trans Orthop Res Soc* 25:1010, 2000.

Chen AC, Temple MM, Ng DM, Richardson CD, DeGroot J, Verzijl N, teKoppel JM, Sah RL: Age-related crosslinking alters tensile properties of articular cartilage. *Trans Orthop Res Soc* 26:128, 2001.

Kim S, Temple MM, Chen AC, Sah RL: Effects of nitric oxide on cartilage homeostasis and chondrocyte viability. *Trans Orthop Res Soc* 26:105, 2001.

Bae WC, Temple MM, Rivard KL, Niederauer GG, Sah RL: Indentation testing of human articular cartilage: sensitivity to indices of degeneration. *Trans Orthop Res Soc* 27:252, 2002.

Rivard KL, Temple MM, Bae WC, Sah RL: Aging-associated biomechanical weakening of human patellar cartilage: relationship to tissue structure and composition. *Trans Orthop Res Soc* 27:400, 2002.

Temple MM, Bae WC, Rivard KL, Amiel D, Coutts RD, Sah RL: Age- and site-associated biomechanical weakening of human articular cartilage of the femoral condyle relationship to cellularity and wear. *Trans Orthop Res Soc* 27:84, 2002. *(Podium presentation at the 48th Annual Meeting of the Orthopaedics Research Society, February 2002, Dallas, TX)*

Temple MM, Masuda K, Pietryla DW, Thonar EJ-MA, Sah RL: Age- and site-associated weakening of human articular cartilage: relationship to collagen denaturation. *Trans Orthop Res Soc* 28:707, 2003.

Nugent GE, Law AL, Wong EG, Temple MM, Bae WC, Chen AC, Kawcak CE, Sah RL: Site- and exercise-related variation in structure and function of articular cartilage from equine distal metacarpal condyle. *Trans Orthop Res Soc* 29:551, 2004.

Schumacher BL, Schmidt TA, Bae WC, Wong VW, Temple MM, Kim TW, Voegtline MS, Chawla K, Chen AC, Bugbee WD, Sah RL: Localization and expression of lubricin/superficial zone protein in human articular cartilage: alterations in osteoarthritis. *Trans Orthop Res Soc* 29:281, 2004.

Temple MM, Jadin KD, Bissar O, Bae WC, Chen MQ, Price JH, Sah RL: Changes in chondrocyte content and organization in human articular cartilage with early degeneration. *Trans Orthop Res Soc* 29:943, 2004.

Temple MM, Xue Y, Chen MQ, Sah RL: IL-1 induces tensile weakening and collagen degradation in cartilage. *Trans Orthop Res Soc* 29:112, 2004. *(Podium presentation at the 50th Annual Meeting of the Orthopaedics Research Society, March 2004, San Francisco, CA)*

Temple M, Bae W, Jadin K, Bissar O, Chen M, Price J, Sah R: Decreased cellularity in the superficial zone of human articular cartilage in early degeneration. *Int Cart Repair Soc* 5, 2004.

# ABSTRACT OF THE DISSERTATION

AGE- AND SITE- ASSOCIATED BIOMECHANICAL WEAKENING
OF HUMAN ARTICULAR CARTILAGE:
RELATIONSHIP TO CELLULARITY, WEAR, MATRIX FRAGMENTATION
AND THE PROGRESSION TO OSTEOARTHRITIS

by

Michele M. Temple

Doctor of Philosophy in Bioengineering
University of California, San Diego, 2005
Professor Robert L. Sah, Chair

During aging, macroscopically normal articular cartilage weakens, with a decrease in tensile stiffness and strength. As cartilage degeneration, often age-associated, progresses to osteoarthritis, cartilage displays classical changes of increased fragmentation of aggrecan and the collagen network with a decrease in tissue proteoglycan content, cell cloning near fissures, hypocellularity in other areas of tissue, and further tensile weakening. While the age-related degenerative changes appear to precede osteoarthritic changes, the exact sequence of pathogenic events is unclear. It was the aim of this dissertation to delineate the extent and time-sequence of these changes to

give insight into the mechanisms underlying age-associated degeneration and osteoarthritis.

In particular, changes in articular cartilage with aging and osteoarthritis may be due primarily to (1) mechanical alteration of the surface, (2) enzymatic degradation of extracellular matrix components, or (3) loss and disorganization of cells. To elucidate the time sequence of such changes, and their relevance to *in vivo* processes, the surface structure, composition, and function of human cartilage samples displaying a normal structure, minor fibrillation (early degeneration, from non-osteoarthritic joints), and moderate fibrillation (from osteoarthritic joints) were analyzed. Age-associated tensile weakening of the superficial zone pointed to a role of surface wear as an initiator in early cartilage degeneration, with cell loss and matrix degradation as downstream events. Tensile weakening in mildly and moderately fibrillated cartilage highlighted the combined roles of surface wear, cell loss, matrix degradation, and collagen network remodeling in the progression of osteoarthritis. Detailed two- and three-dimensional study of the density and organization of chondrocytes of the superficial zone revealed a mild age-associated decrease in cell density and a noteable loss of cells and cell organization in early cartilage degeneration. IL-1-induced enzymatic degradation of articular cartilage was quantified as an initial loss of glycosaminoglycan followed by degradation of the collagen network. Cartilage weakening occurred at a time subsequent to glycosaminoglycan loss and parallel to collagen degradation, suggesting a causal role for collagen degradation in tensile weakening.

The information obtained from these studies gave insight to the role of, and relationship between, cells and matrix in cartilage aging and osteoarthritic disease.

# CHAPTER 1

# INTRODUCTION

## 1.1 General Introduction to the Dissertation

With age, macroscopically normal adult human knee articular cartilage weakens appreciably, evidenced as decreases in the dynamic tensile stiffness and strength of the cartilage matrix [50]. Age-related weakening of cartilage may predispose the knee joint to development of pain, dysfunction, and the classical histopathological features of osteoarthritis (OA). Early degenerative changes in articular cartilage, including an increase in water content, altered cell density and organization, fragmentation of proteoglycans and degradation of the collagen network, may be indicative of changes occurring with age which predispose articular cartilage to mechanical weakening. The site- and depth-dependence, as well as the extent and sequence, of changes underlying age-associated cartilage degeneration and osteoarthritis remain unclear.

*The overall motivation of this dissertation is to further define the causal mechanisms of aging and osteoarthritis in cartilage as related to mechanical dysfunction, by assessing the sequence of events that occur in normal human articular cartilage with aging and characteristics of cartilage that change with the progression*

*to osteoarthritis.* The overall objectives of this work were to: (1) characterize, in normal human articular cartilage of the lateral and medial femoral condyles changes with age in tensile biomechanical properties, density of cells, content of extracellular matrix components, and structural indices of surface wear (Chapter 2), (2) determine if age- and site-matched samples that exhibit early degeneration (a mildly fibrillated surface) and osteoarthritis (moderately fibrillated tissue) display properties distinct from those of normal samples (Chapter 3), (3) characterize the cell density and organization of the superficial zone of normal and mildly degenerate articular cartilage in two and three dimensions (Chapter 4), and (4) in a model of cartilage degeneration, induced by interleukin-1 (IL-1), analyze the time-dependent release of glycosaminoglycan and cleavage and denaturation of collagen, and the relationship of matrix degradation to the tensile weakening of cartilage (Chapter 5).

Chapter 1 begins with a description of the composition, structure and function of normal articular cartilage. This is followed by a discussion of the known changes in tensile biomechanical properties of articular cartilage with age and degeneration. Finally, changes in the cellularity, biochemical content, and structure of articular cartilage with age and degeneration are reviewed which may be associated with changes in the biomechanical function of articular cartilage.

Chapter 2, which has been submitted to the journal *Arthritis and Rheumatism*, addresses changes with age, in macroscopically normal articular cartilage, in the surface structure, cellularity, biochemical composition, and fluorescence associated with advanced glycation endproducts, and the possible relationship to tensile weakening. Results highlighted a role for surface wear and fatigue in the initiation of

cartilage tensile weakening with age and cell loss and matrix degradation as subsequent events involved in the progression of early cartilage degeneration.

Chapter 3 described, in age-matched normal, mildly degenerate, and moderately degenerate articular cartilage, alteration of matrix components and intrinsic fluorescence, structural indices of wear, and cellularity as associated with tensile weakening in early cartilage degeneration. This chapter showed a marked decrease in dynamic and equilibrium tensile properties, with changes in matrix composition, consistent with collagen network degradation and remodeling, as possible instigators of tensile weakening associated with early cartilage degeneration.

Chapter 4 of the dissertation studies in two- and three-dimensions the density and organization of articular chondrocytes of the superficial and transitional zones of Young and Aged cartilage as well as age-matched normal and mildly degenerate articular cartilage. There were mild age-associated decreases and marked degeneration-associated decreases in cell density observed in *en face* views of the cartilage paralleled by an apparent alteration of chondrocyte organization of the superficial zone.

Chapter 5 studied, in a model of matrix degradation induced by IL-1, the time course of loss of glycosaminoglycan and the cleavage and denaturation of collagen molecules, as well as the relationship to the loss of tensile properties. IL-1 induced an initial loss of glycosaminoglycan from the matrix without any affect on tensile properties, but this was followed by degradation of the collagen network and tensile weakening evident as a loss of tensile strength and decreased failure strain, with the

superficial and middle layers being most affected. This form of matrix degradation may play a role in more advanced stages of osteoarthritis.

Chapter 6 discusses the major findings and gives future directions for this work.

## 1.2 Articular Cartilage Function, Composition, and Structure

### 1.2.1 Function

Articular cartilage is a white, glossy connective tissue that covers the ends of long bones within synovial joints. It acts to distribute contact stresses evenly over the surface area of the joint and, because its surface is smooth, lubricated, and wear-resistant, it facilitates joint motion. During normal locomotion, cartilage experiences high loads in tension, compression, and shear. In the knee, the compressive force borne by cartilage of the tibial plateau has been calculated to be up to four times body weight [74]. Tensile and shear forces within cartilage of the knee are a result of frictional forces developed during joint translation, and additional tensile forces result from tension associated with the curvature of the joint surface and weight bearing [16, 98]. In osteoarthritis, disruption of the collagen network is one of the earliest structural changes, and this has been associated with a decrease in the tensile modulus and strength [2] and an increase in water content of the cartilage [8, 10, 55]. However, the changes in biochemical composition and structure of macroscopically normal articular cartilage that may result in the progression to early degeneration or osteoarthritis remain unclear.

### 1.2.2 Chondrocytes

Chondrocytes synthesize, degrade, remodel and maintain the cartilage matrix. The functional unit of cartilage is termed a chondron which consists of chondrocytes surrounded by a pericellular matrix. The pericellular matrix is composed of a fine network of collagen-like fibrillar material and a dense accumulation of proteoglycans, surrounding the chondrocytes in spaces called lacunae. It is through this pericellular matrix that chondrocytes receive and respond to signals which, in turn, can affect the overall cartilage matrix composition and structure (Figure 1.1). Thus, articular cartilage has compositional, structural, and metabolic properties which are interrelated and affect the overall function of the tissue. Cyclic loading, for example, can lead to rapid increases in hydrostatic pressure, matrix deformation, and fluid flow, mechanical stimuli that, depending on the amplitude, can lead to a microenvironment around the chondrocyte that will stimulate protein synthesis [40, 72, 110]. This matrix synthesis can alter the overall physical properties and mechanical response of cartilage to load. A detrimental shift in the loading pattern of a joint may lead to the imbalance of synthesis and degradation of matrix molecules that is associated with the onset of osteoarthritis [99].

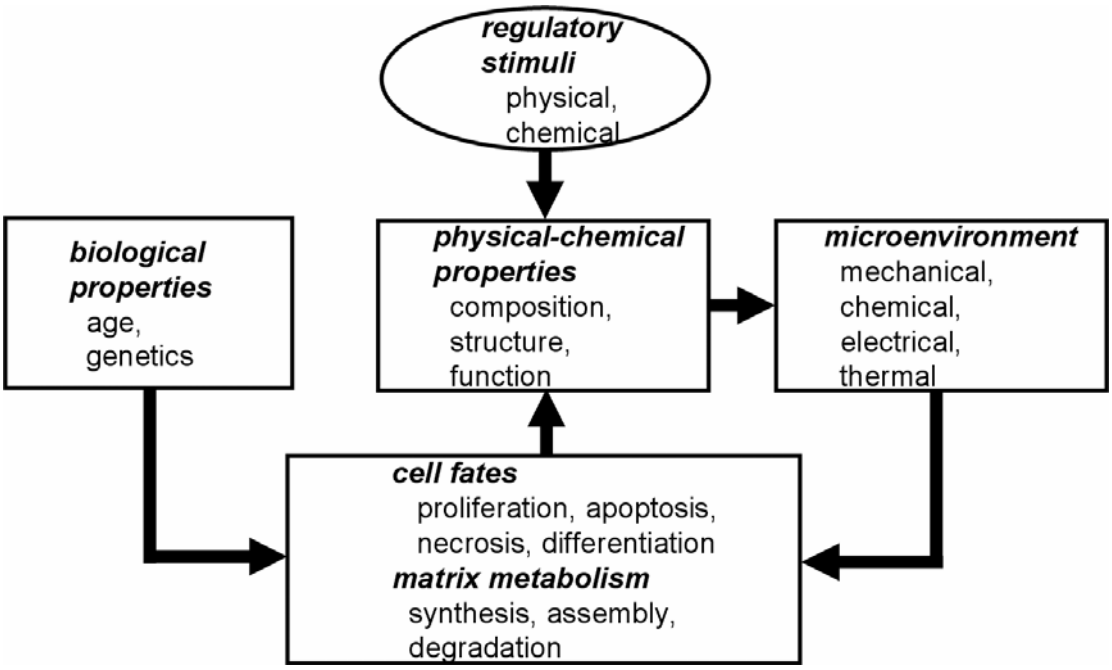



**Figure 1.1:** Interrelationships of cartilage cells, composition, regulatory stimuli, and biological properties.

### 1.2.3 Composition

Adult articular cartilage is composed of a sparse population of cartilage chondrocytes and a fluid-filled extracellular matrix (ECM, Figure 1.2). Articular cartilage is a multiphasic material, composed of a fluid phase of water (68-85% of the wet weight) and electrolytes, and a solid phase of mostly collagen (10-20% of the wet weight), and proteoglycans (5-10% of the wet weight) [19, 55-57, 75, 76]. The multiphasic properties of cartilage endow it with its mechanical properties. Proteoglycans maintain tissue hydration, and because of the high osmotic pressure associated with them, can maintain hydration under considerably high applied loads [55, 56, 59, 104]. The swelling pressure associated with proteoglycans contributes to the equilibrium compressive stiffness of cartilage and is counteracted by the collagen network. The collagen network maintains the tissue integrity of cartilage and withstands high tensile loads generated during weight-bearing and by the swelling tendency of proteoglycans [56].

Collagen is formed by the interaction of three alpha chains. The collagen alpha chain is a polypeptide of repeating subunits of Gly-X-Y, where X is often proline and Y is often hydroxyproline. Hydroxyproline results as a post-translational modification of proline and adds stability to the triple helical configuration. It is present in cartilage only in collagen and is commonly used as a biomarker for collagen content and metabolism [86, 109]. Lysine can undergo hydroxylation, and the hydroxylysine residues are sites for enzymatic collagen crosslinking. It is the unique combination of alpha chains that gives rise to several different types of collagen molecules.

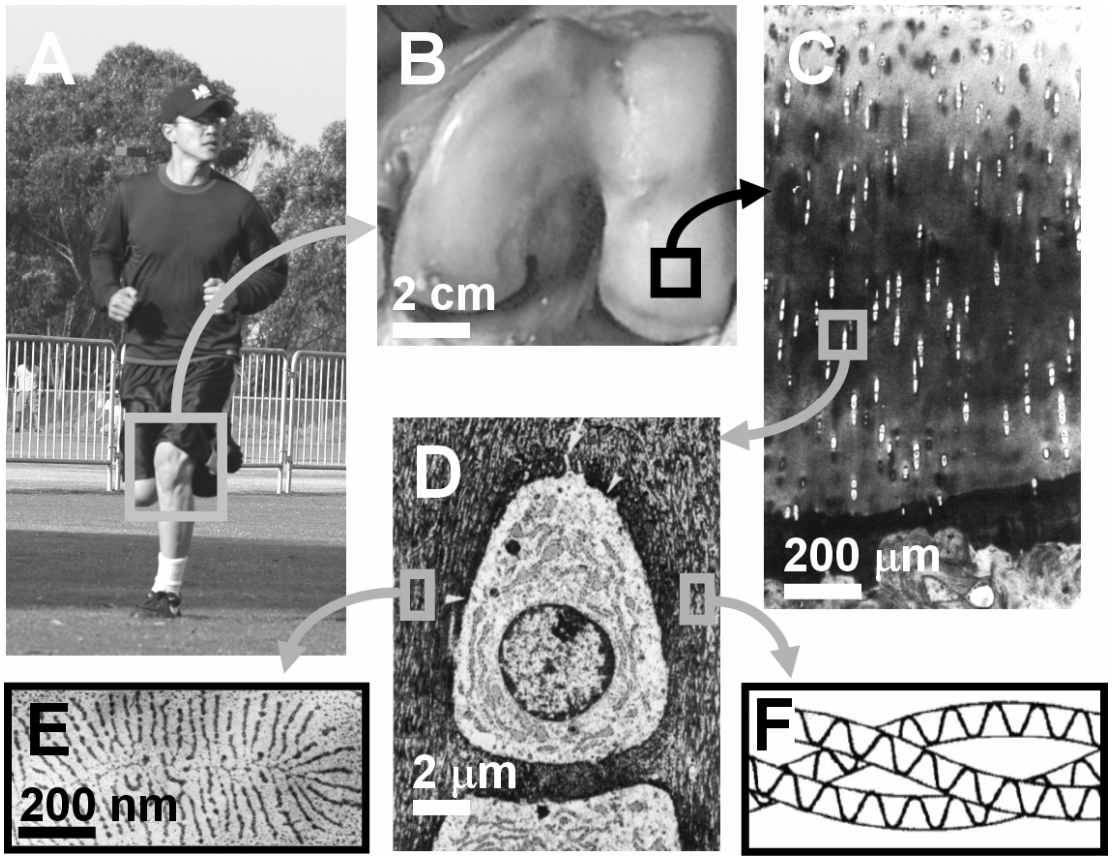



**Figure 1.2:** Articular cartilage composition and length scales. (**A**) Human knee joints are complex structures which include the articulation of the (**B**) distal femur over the proximal tibial plateau (not shown). (**B**) Articular cartilage covers the end of long bones of articulating joints and is comprised of (**C**) a depth-dependent composition of (**D**) a sparse population of chondrocytes and (**E, F**) the matrix constituents, proteoglycans and collagen respectively. Micrographs and collagen schematic were adapted from [18, 30, 46].

The collagen composition of adult articular cartilage is 90-98% type II collagen [19, 32]. Smaller amounts of collagen types III, VI, IX, X, XI, XII, and XIV are also present [17, 32]. Most collagen fibers in cartilage, are heterotypic, with a core of type XI collagen surrounded by a sheath of type II collagen and molecules of type IX collagen on the surface (Figure 1.3) [68]. Types II and XI collagen are fibril forming collagens that contain a large central triple helical domain, stabilized by hydrogen bonds, with nonhelical N- and C- propeptide domains. A type II collagen molecule is a homotrimer formed by the polymerization of three identical α1(II) chains, while a type XI collagen molecule is a heterotrimer of a1(XI), a2(XI), and a3(XI) chains. They form quarter staggered fibrillar aggregates once their propeptide domains are cleaved. Type XI collagen, however, retains its N-propeptide sequences [73] which may prevent lateral growth of fibrils and regulate fibril diameters [33, 68]. Type IX collagen is a non-fibrillar collagen that does not form quarter staggered fibrils but crosslinks to the surface of type II collagen fibrils as seen by rotary shadowing, immunolocalization, and electron microscopy [48, 69, 105]. Type IX collagen is a heterotrimer of three distinct alpha chains that form three triple helical domains and four non-helical domains. It has six possible cross-linking sites that can interact with type II collagen fibrils and other type IX collagen molecules. It has a single site on the a2(IX) chain for covalent attachment of a chondroitin sulfate glycosaminoglycan chain, although the size of the chain can vary and is considered a minor component of cartilage ECM [27]. With such structural characteristics it is thought to serve as an intermediary in collagen fibril and proteoglycan associations, perhaps regulating fibrillogenesis and fiber diameter [33].

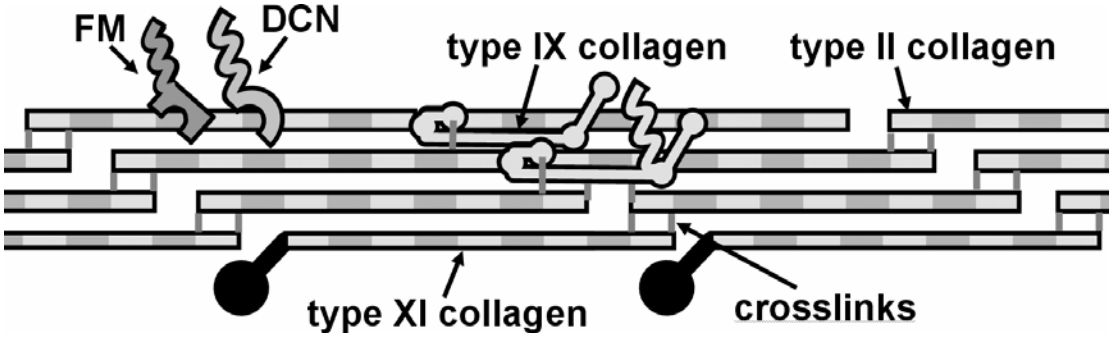



**Figure 1.3:** Schematic of the collagen II, IX, and XI heterotrimer composing a cartilage collagen fiber. Type II collagen fibrils will form a sheath around type XI collagen fibrils. Type IX collagen molecules are proposed to fold and interact with molecules on the surface of collagen II fibrils to accommodate cross-linking interactions and potentially assist in fibril-to-fibril crosslinks in a network. Decorin (DCN) and fibromodulin (FM) are small proteoglycans which can also bind to the surface of collagen II fibrils and may play a role in controlling fibril diameter and collagen network interactions.

Other collagen types, found in small amounts in cartilage or in developmental or pathological conditions, include types III, VI, X, XII, and XIV. Type III has been detected immunochemically in normal and osteoarthritic human articular cartilage and has been shown to co-localize with type II collagen to the surface of banded fibrils. However, it is unknown if its synthesis by chondrocytes is a remodeling or repair response [32]. Type VI collagen forms beaded microfilaments in the pericellular region around chondrocytes [85] and has several RGD sequences in each chain [4], implying that it has cell attachment properties and may play a role in cellular interactions with the ECM. Type X collagen is formed predominantly by hypertrophic chondrocytes during the process of endochondral ossification and is found in the deep calcified layer of mature articular cartilage [77]. It has been used as a marker for normal and aberrant bone formation and has been localized in osteoarthritic joints [45, 107]. Collagen types XII and XIV are non-fibrillar collagens, found in addition to collagen IX, at the surface of collagen fibrils [32].

Collagen fibrils are stabilized by inter- and intra-molecular crosslinks, formed by enzyme-mediated reactions [35] or by nonenzymatic glycation (NEG) reactions [90]. The crosslinks may directly contribute to the mechanical properties of the tissue. The principal cross-linking residues in mature type II collagen are hydroxylysyl pyridinoline (HP) residues [34, 35] which form enzyme-mediated crosslinks. The formation of aldehydes is catalyzed by the enzyme lysyl oxidase in each of the telopeptide domains of the type II collagen molecule. These interact with hydroxylysine residues at two specific triple helical sites in adjacent molecules to form HP crosslinks. They can be quantified by their natural fluorescence [91, 102]. There is little variation in the quantity of the HP crosslinks as the tissue ages [7]. NEG products accumulate in cartilage due to the long life of collagen molecules. They form

from the condensation of a sugar aldehyde or ketone with lysine or hydroxylysine into stable amadori products, which further react to form fluorescent advanced Maillard (or browning) products [90]. The fluorescent characteristic of these crosslinks can also be used for their quantification [36, 62, 103] and localization in normal and osteoarthritic cartilage [37]. The quantity of pentosidine crosslinks, a small fraction of all NEG products, increases slowly with age in articular cartilage [7, 82, 103] and is thought to contribute to increasing matrix stiffness or brittleness [106].

The cartilage ECM, in addition to the extensive collagen network, is composed of a proteoglycan gel that has a high fixed charge density, which leads to a large osmotic swelling pressure by the Donnan effect [56]. In a free swelling solution, proteoglycans will expand to five times their volume in tissue [41]. The large aggregating proteoglycan, aggrecan, makes up 80-90% of the proteoglycans in cartilage [19, 56]. Aggrecan is composed of a protein core organized into three globular domains spaced around two regions dense with glycosaminoglycan side chains (Figure 1.4) [29, 42]. At the amino terminal is the G1 globular domain that specifically interacts with hyaluronan. Next to the G1 globular domain, in the direction of the carboxy terminal, are a short peptide sequence, known as the interglobular domain, and the G2 globular domain. The G1 and G2 domains form a region that could serve to immobilize the high charge density proteoglycans through linkage to the fibrillar network [79]. Following the G2 domain are the keratan sulfate and chondroitin sulfate-rich regions and the G3 globular domain at the carboxy terminal end. In all, there are approximately 100 chondroitin sulfate and 50 keratan sulfate side chains attached to a molecule of aggrecan, each of which has about 100 negatively charged carboxy and sulfate groups, endowing it with a high density of negative charge [42]. Molecules of aggrecan interact with hyaluronan in an interaction

stabilized by link protein [20, 67, 84] to form large aggregates. Less than ten percent of the proteoglycans in articular cartilage are the small proteoglycans, decorin, biglycan, and fibromodulin [89], which can bind to the surface of collagen fibrils (Figure 1.3) and are thought to play a role in controlling fibril diameter [80]. It is the interactions of the cartilage cells and matrix constituents which dictate how the cartilage will respond to mechanical loading. Alteration of the cellular synthesis of these constituents or the molecular structure, organization, or overall quantities of the resident matrix molecules may lead to the progression of cartilage degeneration.

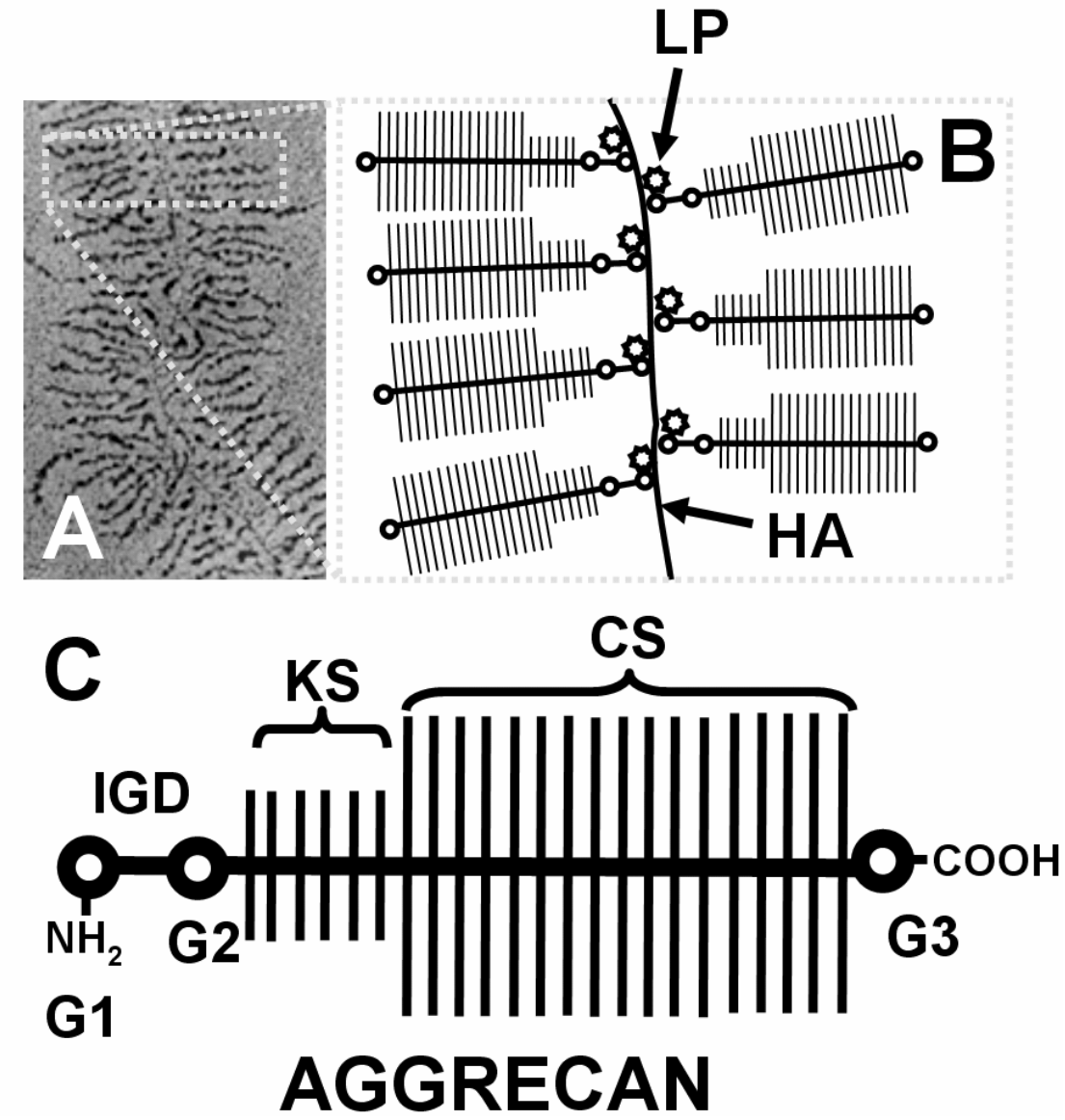



**Figure 1.4:** Structure of the large aggregating proteoglycan, aggrecan. (**A**) Large aggregating proteoglycans are visible in electon micrographs [18]. (**B**) Molecules of aggrecan will bind to hyaluronan (HA) through an interaction stabilized by link protein (LP). (**C**) An aggrecan monomer is composed of three globular domains (G1, G2, and G3) that are interspersed about an interglobular domain (IGD) and regions rich in keratan sulfate (KS) and chondroitin sulfate (CS) glycosaminoglycans.

### 1.2.4 Ultrastructure

The organization of cells and matrix constituents varies with depth from the articular surface, and for descriptive purposes has been subdivided into zones aligned parallel to the articular surface [25, 46, 66]. The superficial, middle (or transitional), and deep zones are about 10-20%, 40-50% and 40% of the full thickness of uncalcified cartilage, respectively [46, 66], and lay atop a layer of calcified cartilage and subchondral bone (Figure 1.5). Each articular cartilage zone has a unique organization and composition of cells and matrix constituents as well as mechanical properties [52, 95]. In the superficial zone, there is a high density of articular chondrocytes that are discoid in shape with their long axis oriented parallel to the articular surface [46]. Collagen fibers are oriented tangential to the articular surface and are relatively small in diameter [23, 65, 94, 108]. In the middle zone, chondrocytes are spheroidal and less dense, and collagen fibers are larger in diameter and randomly oriented. In the deep zone, the cells are spheroidal in shape and have a columnar organization where collagen fibers are large in diameter and oriented perpendicular to the subchondral bone. The composition of matrix constituents in each zone is unique, with collagen and water contents decreasing slightly [55, 58] and GAG content increasing with depth [58]. It is the distinct organization and composition of articular cartilage that contribute to its depth-dependent tensile and compressive mechanical properties [52, 95].

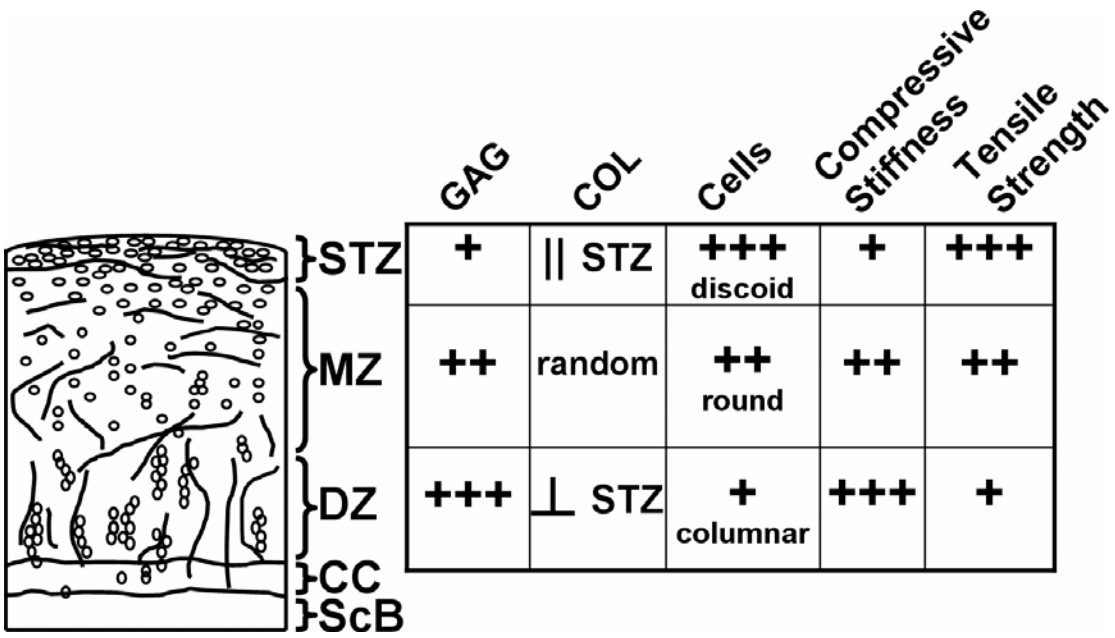



**Figure 1.5:** The zonal variation of cells, extracellular matrix components, and cartilage mechanical properties. Glycosaminoglycan (GAG) content, collagen (COL) network orientation, cell density and morphology, and mechanical properties are described for the superficial tangential zone (STZ), the middle zone (MZ), and the deep zone (DZ) of uncalcified articular cartilage, which are atop a layer of calcified cartilage (CC) and subchondral bone (ScB).

## 1.3 Alteration of Articular Cartilage Biomechanical Properties with Age and Degeneration

In 1982, Kempson found that the tensile strength and stiffness of articular cartilage diminish with age [50]. However, data from this study was averaged over multiple sites on the knee joint, and although the cartilage was judged to be macroscopically normal, it is unknown if the cartilage exhibited histological or biochemical signs of disease. The mechanical properties of articular cartilage are site- and depth-dependent [2, 3, 51, 52]. These properties are also dependent on the collagen network organization at the surface (noted by splitlines) [52] and the structure and content of collagen and glycosaminoglycans in the ECM [2, 52]. It is unknown how tensile weakening with age is localized in the knee joint or with depth from the articular surface or how this affects or is affected by the ECM composition or stage of disease.

Analysis of the natural sequence of events in detail may clarify potential pathogenic mechanisms. Biomechanical weakening with age may result in an increased predilection for development of osteoarthritis. Such weakening may reflect cell death [87, 88, 100] and an inability of the remaining cells to maintain tissue homeostasis (Figure 1.6A). Or, such weakening may directly reflect mechanical wear [63], with physical disruption of collagen fibers of the articular surface precipitating further cartilage degeneration (Figure 1.6B). Finally, enzymatic degradation and loss of the collagen network and proteoglycan aggregates [5, 28], may be a primary event leading to degeneration, with altered molecular structure of matrix molecules leading to an inferior cartilage matrix (Figure 1.6C).

These three possible mechanisms of biomechanical weakening with age may lead to further cartilage degeneration and ultimately osteoarthritic disease. In

particular, an initiator of mechanical weakening may be mechanically-induced alteration of the articular surface and matrix structure. As the collagen fibers of the surface are disrupted, there may be an associated cellular response and enzymatically-induced matrix degradation. Mechanical weakening may also be induced by cell death and disorganization. The resulting alteration of matrix metabolism may further progress to disruption of the articular surface and matrix degradation. Mechanical weakening may also originate with enzymatically-induced alteration of the ECM components. Fragmentation of proteoglycan aggregates and cleavage and denaturation of the collagen network may result from chondrocyte and synoviocyte production of pro-inflammatory cytokines and proteases. The altered microenvironment around the cells and altered response of the matrix to mechanical load could result in cell death and surface wear.

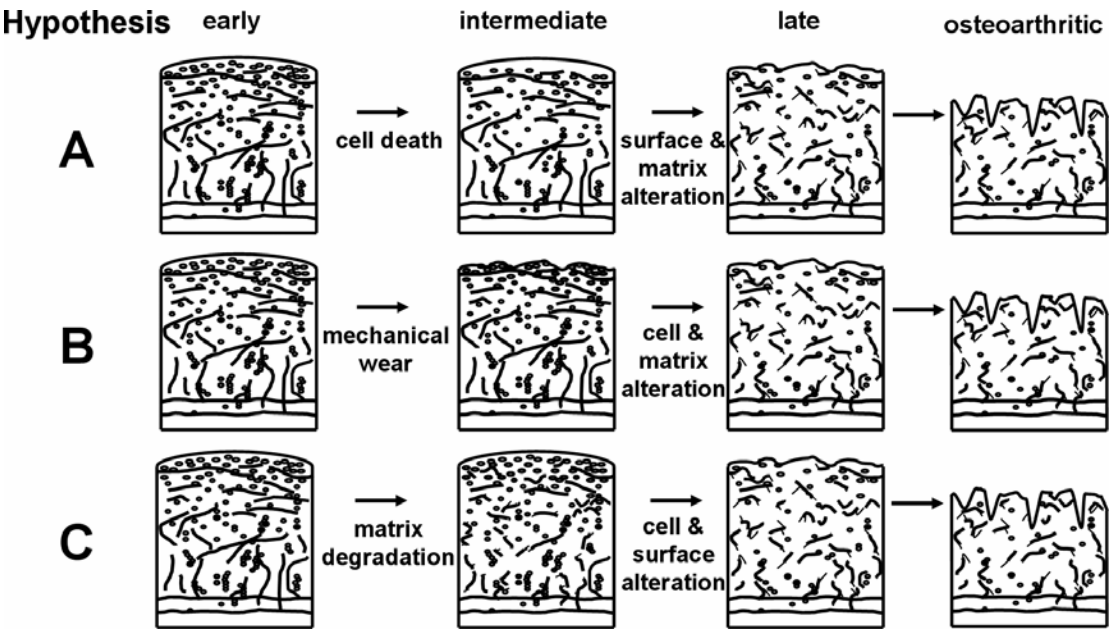



**Figure 1.6:** Proposed mechanisms through which articular cartilage progresses from degeneration to an osteoarthritic disease state. (**A**) Cells could undergo some form of cell death resulting in altered matrix metabolism and mechanical function. Subsequently, there may be alteration of the articular surface and matrix components. (**B**) The articular surface could initially be roughened, altering the mechanical environment around the cells, subsequently resulting in cell death and alteration in matrix content and structure. (**C**) Enzymatically-induced matrix degradation may occur prior to significant loss of cells or alteration of the articular surface.

## 1.4 Alteration of Cell Density and Organization of Articular Cartilage with Age and Degeneration

The decrease in tensile mechanical properties may result from cell death and age-associated alteration of cell density and organization. From approximately forty years of age onward, the cell density of the superficial zone decreases with age ~6.3% per decade in human femoral condylar cartilage [87], but this may be dependent on anatomical location and depth from the articular surface [100]. This analysis has traditionally been done by cell counting in vertical sections of cartilage and has been suggested to be an artifact due to the loss of cell nuclei during histological processing [1]. The cellularity of fibrillated cartilage is even more sparse [70, 88] and it is unknown if this is a consequence of cell death or physical loss of cartilage. However, this decrease in cell density may increase the matrix domain or mean matrix volume controlled by a single chondrocyte [47], making remodeling activities low in the far removed interterritorial matrix and cartilage repair problematic.

Chondrocyte organization is altered with the progression of disease. In osteoarthritic cartilage, there is a loss of chondrocytes near the surface with large cell clusters appearing at sites of severe fibrillation and fissuring [70, 88]. Cell clusters, in vertical histological sections of fibrillated cartilage, increase in number but decrease in size with age [70]. This cell clustering may indicate a response by articular chondrocytes to replicate and repair tissue that, in combination with an age-associated decrease in the proliferative activity, is impaired. While large clusters may be pathologic in nature, small cell clusters may be observed in normal cartilage oriented parallel to the surface in ankle cartilage [96] or vertically in the deeper zones of knee joint cartilage [66], though this may be site-dependent. Chondrocytes are required for the normal metabolic homeostasis of articular cartilage, and a pattern of loss and

disorganization in this cellularity may result in the altered matrix structure and biomechanical failure in osteoarthritis.

## 1.5 Alteration of Articular Cartilage Surface Structure with Age and Degeneration

Alterations in cartilage surface structure and histopathological indices are hallmarks of degeneration. Traditionally, the articular cartilage surface has been characterized macroscopically by semi-quantitative grading schemes with varying degrees of detail to distinguish between cartilage with an intact articular surface and cartilage showing morphological signs of disintegration (Figure 1.7) [25, 54, 78, 81, 97, 111]. In 1949, Collins described the macroscopic progression of osteoarthritis in four stages (Table 1.1 [25]). Grade 1 osteoarthritis is confined to the superficial zone, where there may be mild flaking or fibrillation. In Grade 2 osteoarthritis, there is deep fibrillation and loss of cartilage substance. In Grades 3 and 4 stages of osteoarthritis, there is loss of cartilage and exposure of bone with varying amounts of contour remodeling, fibrosis of the joint capsule and synovial membrane, and osteophytes. This macroscopic appearance can vary from joint to joint and even from area to area within a single joint [25, 38]. In the knee joint, degenerative changes occur earlier and are more rapidly progressive on the tibial and patellar facets [11] and also more prevalent and more severe in the medial than the lateral facet (Figure 1.8) [11, 25]. In addition, there is a higher incidence and severity of macroscopic cartilage destruction in the knee joint with age [11, 25], making it difficult to distinguish between age-associated and disease-associated events in the progression of cartilage degeneration.

The origins of articular surface fibrillation are unknown and have been hypothesized to be due to adhesive wear, abrasive wear, or fatigue failure, alone or in

combination [66, 101]. Adhesive wear occurs when surfaces sliding against each other under load have slight projections that adhere. To continue motion, the adhesions are sheared and material may be removed from one or both surfaces. Abrasive wear occurs when slight projections on a surface or between surfaces cause removal of particles from the opposing surface. While adhesive and abrasive wear can result in the loss of wear fragments from one or both surfaces, fatigue wear can occur without loss of material. Fatigue wear occurs when cyclically varying stresses cause fatigue fractures at, or close to, a surface, making it more likely that particles will detach and surface failure will occur. The repeated stress cycles can cause the surface to fail at stresses well below the tensile strength of cartilage. In addition, fatigue of the material may be something unobservable until the point at which the material fails. These forms of wear can be difficult to observe because the rate of wear in healthy joints under normal conditions is low and because cartilage is a self-renewing material [101].

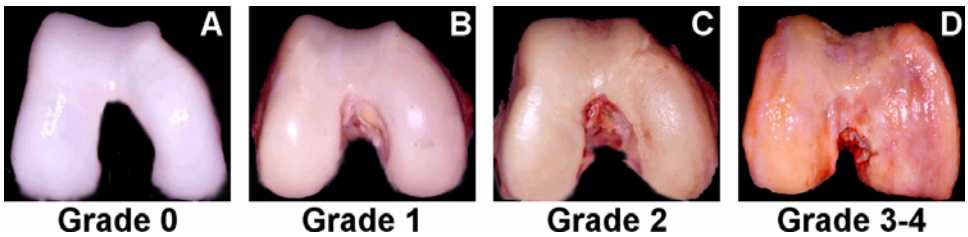



**Figure 1.7:** Gross macroscopic images of the distal femur at various stages of osteoarthritic disease. The macroscopic surface appearance of cartilage was graded as normal (Grade 0, **A**), having mild fibrillation (Grade 1, **B**), having overt cartilage fibrillation (Grade 2, **C**), and full thickness cartilage erosion (Grade 3-4, **D**) according to the Collins grading scheme [25].

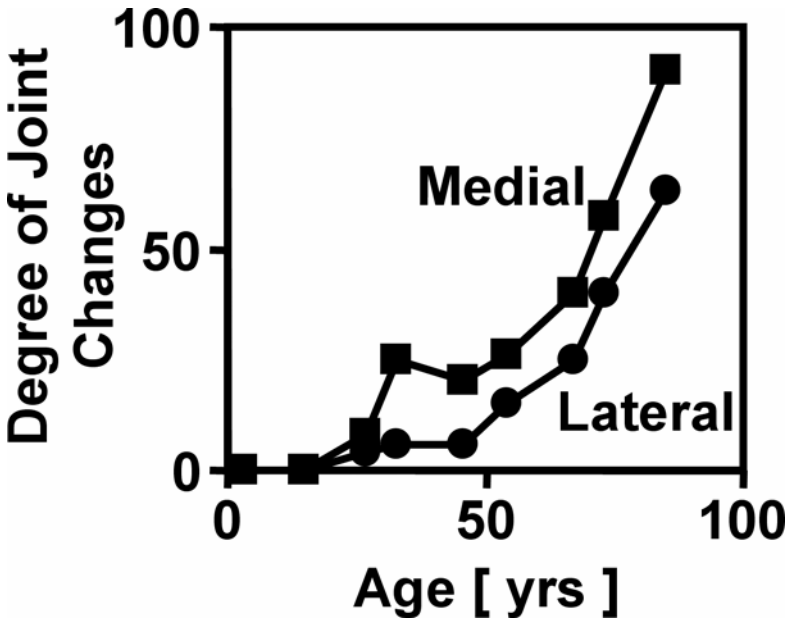



**Figure 1.8:** Severity of degenerative cartilage changes in the medial and lateral femoral condyle adapted from [11]. Cartilage of the medial and lateral femoral condyles were graded according to a semi-quantitative grading scheme for slight unevenness of the articular surface (25), superficial fibrillation and small erosions (50), extensive ulceration (75), and complete erosion with exposure and eburnation of subchondral bone (100).

| GRADE | CHARACTERISITCS |
|---|---|
| 0 | ▪ Smooth, intact articular surfaces<br>▪ Normal capsule, synovial membrane & articular contour |
| 1 | ▪ Superficial, tangential flaking, mild fibrillation, shallow pits/grooves, small blisters beneath surface in areas of greatest pressure/movement<br>▪ Normal capsule, synovial membrane & articular contour |
| 2 | ▪ Deep fibrillation, notable loss of cartilage in areas of greatest pressure/movement<br>▪ Superficial, tangential flaking, mild fibrillation, shallow pits/grooves, small blisters beneath surface in surrounding areas<br>▪ Early marginal hyperplasia, beading or ridging along edges of surfaces most affected<br>▪ No gross remoulding of bone ends |
| 3 | ▪ Total loss of cartilage; exposure/eburnation of bone in pressure/movement areas<br>▪ Fibrillation & flaking of remaining cartilage<br>▪ Osteophytes at margins<br>▪ Alteration of articular contour<br>▪ Schlerosis of subchondral bone<br>▪ Fibrosis of capsule<br>▪ Increase in villosity of synovial membrane |
| 4 | ▪ Complete loss of cartilage from large areas<br>▪ Eburnation of exposed bone<br>▪ Unevenness of surface<br>▪ Prominent marginal osteophytes & spurs & epiarticular osteophytes<br>▪ Remodeling of contour of bone end<br>▪ Fibrosis of capsule & synovial membrane |

**Table 1.1:** Gross morphological features of osteoarthritis and a scheme for the pathological grading of OA [25].

Recent techniques have been developed to better understand the changes at the articular surface with aging and degeneration in macroscopic and microscopic views. Application of India ink to the articular surface highlights regions and patterns of cartilage fibrillation (Figure 1.9). One technique quantifies the ink-staining intensity of such surfaces with a digital video imaging technique [21]. India-ink staining of the surface of articular cartilage has shown that there is an increase in degeneration-affected areas in a joint with age [64]. Scanning electron microscopy has shown that macroscopically normal cartilage has increasing degrees of fibrillation with age [24]. Vertical histological full-thickness cartilage sections can also be analyzed microscopically for structural details. A histopathological grading scheme can be applied to give a semi-quantitative score to the severity of degeneration and OA (Table 1.2 [54, 97]). A histomorphometric method of assessing cartilage surface roughness allows quantitation of structural disruption of the cartilage surface [39]. Applying these methods to normal cartilage during aging and various stages of disease would give insight to the changes occurring at the articular surface which may predispose cartilage to further matrix degradation and alteration of cellular organization.

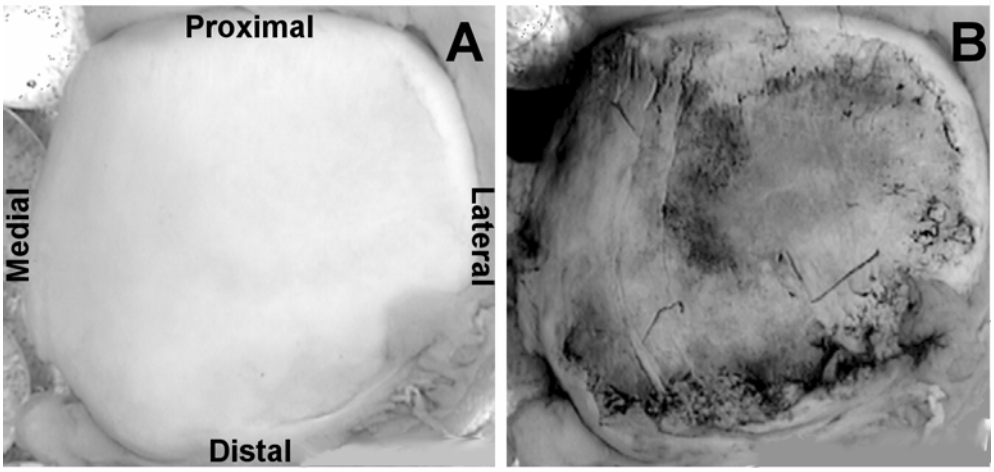



**Figure 1.9:** Application of India ink to highlight regions of cartilage degeneration of the patella. India ink is trapped in regions of fibrillated cartilage allowing obvious detection of cartilage degeneration (**B**) that might otherwise be difficult to observe (**A**).

| Grade | Description | Criteria | Note |
|---|---|---|---|
| **GROSS CHARACTERISTICS** | | | |
| 0 | Normal | Macroscopically normal, slightly rough | |
| 1 | Mild | Extensively roughened, not eroded | |
| 2 | Moderate | Partially eroded | |
| 3 | Severe | Full thickness erosion | |
| **MICROSCOPIC CHARACTERISTICS** | | | |
| Surface Irregularity | | | An irregularity is an unevenness, or interruption by clefts or cracks. |
| 0 | None | | |
| 1 | Mild | ≤ 50% by length is irregular | |
| 2 | Advanced | > 50% irregular | |
| Vertical Clefts to transitional zone | | | A vertical cleft in transitional and radial zones was >45° from the surface and >50 and 300μm deep, respectively. Wide: >20 μm Medium: 5-20 μm Narrow: 0-5 μm |
| 0 | None | | |
| 1 | Mild | 1-3 wide, 1-6 medium, or 1-9 small / 10mm$^2$ | |
| 2 | Advanced | More clefts than above or > 10% area tissue loss in transitional zone | |
| Vertical Clefts to radial zone | | | |
| 0 | None | | |
| 1 | Mild | 1-3 wide, 1-6 medium, or 1-9 small / 10mm$^2$ | |
| 2 | Advanced | More clefts or > 10% area tissue loss in radial zone | |
| Transverse Clefts | | | A transverse cleft was <45° from the surface reaching >50μm deep. |
| 0 | None | | |
| 1 | Mild | > 1 cleft, without tissue loss | |
| 2 | Advanced | >20% area tissue loss | |
| Cloning | | | Clones were ≥4 cells in contact, not in a line. Small: 0-9 cells Medium: 10-15 cells Large: >15 cells |
| 0 | None | | |
| 1 | Mild | 1-2 large, 1-4 medium, or 1-6 small / mm$^2$ | |
| 2 | Moderate | 3-4 large, 5-8 medium, or 7-12 small / mm$^2$ | |
| 3 | Advanced | >4 large, >8 medium, or >12 small / mm$^2$ | |
| Hypocellularity | | | Normal cellularity was defined as 311 cells/mm$^2$ [70]. |
| 0 | None | >233 cells/mm$^2$ | |
| 1 | Mild | 155-233 cells/mm$^2$ | |
| 2 | Moderate | 78-155 cells/mm$^2$ | |
| 3 | Advanced | <78 cells/mm$^2$ | |
| **COMPOSITIONAL CHARACTERISTICS** | | | |
| Glycosaminoglycan Content (GAG) | | | Normal GAG content was 2.1% ww [6]. |
| 0 | Normal | >1.6% of wet weight (ww) | |
| 1 | Slight loss | 1.1-1.6% ww | |
| 2 | Moderate loss | 0.5-1.1% ww | |
| 3 | Severe loss | <0.5% ww | |

**Table 1.2:** A histopathological grading scheme for the semi-quantitative analysis of the severity of cartilage degeneration adapted from [6, 54, 97]. Vertical histological sections are examined for the above characteristics. Values of 0-2 or 0-3 are assigned for each characteristic and summed to provide an overall histopathology score.

## 1.6 Alteration of Articular Cartilage Composition with Age and Degeneration

Mechanical dysfunction of cartilage is due to an imbalance in the normal mechano-osmotic force balance in free-swelling and loaded tissue. The swelling of cartilage, a hallmark of osteoarthritis, results from the osmotically active proteoglycan molecules swelling within a degraded collagen network [15, 55, 60]. Increased cleavage and denaturation of collagen molecules has been observed in OA tissue [9, 28, 43, 44]. Once laid down during development, there appears to be little capacity for articular chondrocytes to recapitulate the overall collagen architecture if the mature tissue is injured or goes through advanced stages of proteolysis. In addition, in normal healthy cartilage, the turnover of collagen is slow [56] and collagen synthesis and incorporation into the collagen network may occur at a rate that is unable to accommodate cartilage repair.

Proteoglycan content and structure is also important in the overall function of articular cartilage. Proteoglycan aggregates (aggrecan) are altered in structure [93] with breakdown products present in synovial fluid of patients with OA [53, 92]. With age, there is increasing heterogeneity in proteoglycans, as a result of varying degrees of proteolytic attack at the carboxy terminus. There is a sharp decrease in intact G3 domain after 30 years of age [31] which may be due to increased proteolytic activity or a decrease in the turnover rates of aggrecan [14].

The degradation of proteoglycans and collagen with age and osteoarthritis may be mediated by cytokines and proteases. The inflammatory cytokine, interleukin-1 (IL-1), has been linked to the induced cleavage and denaturation of type II collagen [43]. IL-1 induces increased collagenase synthesis and secretion from a variety of cells including synovial cells, chondrocytes, and fibroblasts [26, 49, 71]. Collagenases are a

class of enzymes capable of site specific cleavage of helical collagen, and belong to a family of zinc-dependent endopeptidases called matrix metalloproteinases (MMPs). Some studies have shown enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage and have suggested that this is related to increased expression of collagenase-3 (MMP-13) and neutrophil collagenase (MMP-8) [12]. IL-1 is also known to induce proteoglycan degradation [13, 83]. IL-1 can induce synthesis of matrilysin (MMP-7) which vigorously degrades proteoglycan and aggrecanases which are responsible for a specific cleavage that releases large C-terminal fragments from aggrecan [61]. It is unknown if this matrix degradation is site- and depth-dependent or if it is a causal factor in mechanical weakening with age.

## 1.7 Modification of the Collagen Network

Tensile weakening with age and the onset of osteoarthritic disease may also be a result of non-enzymatic glycation modifications to the collagen network. Increased water content of degenerate cartilage, a hallmark of osteoarthritis, is associated with the inability of a weakened collagen network to constrain cartilage swelling [8, 10, 55, 58, 60]. Non-ezymatic glycation resulting in increased crosslinking has been hypothesized to result in a weakened collagen network. Advanced glycation end products (AGEs), including pentosidine, in articular cartilage accumulate with age [7, 103]. Pentosidine crosslinks, in particular, have been associated with a decrease in instantaneous deformation and increased collagen stiffening [7], which is thought to indicate a more brittle and fragile collagen network. While the mechanical effects of pentosidine crosslinks have been studied extensively in the past, it is, as yet, unclear if

the extent of glycation that occurs with aging is sufficient to precipitate the weakening (lowered strength and ramp modulus) of cartilage seen in aging or arthritis [22].

## 1.8 References


1. Aigner T, Hemmel M, Neureiter D, Gebhard PM, Zeiler G, Kirchner T, McKenna L: Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritic human articular knee cartilage. *Arthritis Rhuem* 44:1304-12, 2001.

2. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH: Tensile properties of human knee joint cartilage: I. influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus. *J Orthop Res* 4:379-92, 1986.

3. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH: Tensile properties of human knee joint cartilage: II. correlations between weight bearing and tissue pathology and the kinetics of swelling. *J Orthop Res* 5:173-86, 1987.

4. Aumailley M, Mann K, von der Mark H, Timpl R: Cell attachment properties of collagen type VI and Arg-Gly-Asp dependent binding to its alpha 2(VI) and alpha 3(VI) chains. *Exp Cell Res* 181:463-74, 1989.

5. Bader DL, Kempson GE, Barrett AJ, Webb W: The effects of leucocyte elastase on the mechanical properties of adult human articular cartilage in tension. *Biochim Biophys Acta* 677:103-8, 1981.

6. Bae WC, Temple MM, Amiel D, Coutts RD, Niederauer GG, Sah RL: Indentation testing of human cartilage: sensitivity to articular surface degeneration. *Arthritis Rheum* 48:3382-94, 2003.

7. Bank RA, Bayliss MT, Lafeber FPJG, Maroudas A, TeKoppele JM: Ageing and zonal variation in post-translational modification of collagen in normal human articular cartilage. The age-related increase in non-enzymatic glycation affects biomechanical properties of cartilage. *Biochem J* 330:345-51, 1998.

8. Bank RA, Krikken M, Beekman B, Stoop R, Maroudas A, Lafeber FPJG, Te Koppele JM: A simplified measurement of degraded collagen in tissues: application in healthy, fibrillated and osteoarthritic cartilage. *Matrix Biol* 16:233-43, 1997.

9. Bank RA, Krikken M, Lafeber FPJG, Te Koppele JM: A simplified measurement of in situ denatured collagen in healthy and osteoarthritic cartilage. *Trans Orthop Res Soc* 21:750, 1996.

10. Bank RA, Soudry M, Maroudas A, Mizrahi J, TeKoppele JM: The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. *Arthritis Rheum* 43:2202-10, 2000.

11. Bennett GA, Waine H, Bauer W. Changes in the knee joint at various ages with particular reference to the nature and development of degenerative joint disease. New York: The Commonwealth Fund; 1942.

12. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chan J, Van Wart H, Poole AR: Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* 99:1534-45, 1997.

13. Billinghurst RC, Wu W, Ionescu M, Reiner A, Dahlberg L, Chen J, van Wart H, Poole AR: Comparison of the degradation of type II collagen and proteoglycan in nasal and articular cartilages induced by interleukin-1 and the selective inhibition of type II collagen cleavage by collagenase. *Arthritis Rheum* 43:664-72, 2000.

14. Bolton MC, Dudhia J, Bayliss MT: Age-related changes in the synthesis of link protein and aggrecan in human articular cartilage: implications for aggregate stability. *Biochem J* 337 ( Pt 1):77-82, 1999.

15. Bonassar LJ, Frank EH, Murray JC, Paguio CG, Moore VL, Lark MW, Sandy JD, Wu JJ, Eyre DR, Grodzinsky AJ: Changes in cartilage composition and physical properties due to stromelysin degradation. *Arthritis Rheum* 38:173-83, 1995.

16. Broom ND, Myers DB: A study of the structural response of wet hyaline cartilage to various loading situations. *Connect Tissue Res* 7:227-37, 1980.

17. Bruckner P, van der Rest M: Structure and function of cartilage collagens. *Micros Res Tech* 28:378-84, 1994.

18. Buckwalter JA, Kuettner KE, Thonar EJ: Age-related changes in articular cartilage proteoglycans: electron microscopic studies. *J Orthop Res* 3:251-7, 1985.

19. Buckwalter JA, Mankin HJ: Articular cartilage. Part I: tissue design and chondrocyte-matrix interactions. *J Bone Joint Surg Am* 79-A:600-11, 1997.

20. Buckwalter JA, Rosenberg LC, Tang L-H: The effect of link protein on proteoglycan aggregate structure: an electron microscopic study of the molecular architecture and dimensions of proteoglycan aggregates reassembled from the proteoglycan monomers and link proteins of bovine fetal epiphyseal cartilage. *J Biol Chem* 259:5361-3, 1984.

21. Chang DG, Iverson EP, Schinagl RM, Sonoda M, Amiel D, Coutts RD, Sah RL: Quantitation and localization of cartilage degeneration following the induction of osteoarthritis in the rabbit knee. *Osteoarthritis Cartilage* 5:357-72, 1997.

22. Chen AC, Temple MM, Ng DM, DeGroot J, Verzijl N, TeKoppele JM, Sah RL: Induction of advanced glycation endproducts alters tensile properties of articular cartilage. *Arthritis Rheum* 46:3212-7, 2002.

23. Clark JM: Variation of collagen fiber alignment in a joint surface: a scanning electron microscope study of the tibial plateau in dog, rabbit, and man. *J Orthop Res* 9:246-57, 1991.

24. Clark JM, Simonian PT: Scanning electron microscopy of "fibrillated" and "malacic" human articular cartilage: technical considerations. *Microsc Res Tech* 37:299-313, 1997.

25. Collins DH. The pathology of articular and spinal disease. London: Arnold; 1949.

26. Dayer JM, de Rochemonteix B, Burrus B, Demczuk S, Dinarello CA: Human recombinant interleukin 1 stimulates collagenase and prostaglandin E2 production by human synovial cells. *J Clin Invest* 77:645-8, 1986.

27. Diab M, Wu JJ, Eyre DR: Collagen type IX from human cartilage: a structural profile of intermolecular cross-linking sites. *Biochem J* 314:327-32, 1996.

28. Dodge GR, Poole AR: Immunohistochemical detection and immunochemical analysis of type II collagen degradation in human normal rheumatoid, and osteoarthritis articular cartilage and in explants of bovine articular cartilage cultured with interleukin 1. *J Clin Invest* 83:647-61, 1989.

29. Doege K, Sasaki M, Horigan E, Hassell JR, Yamada Y: Complete primary structure of the rat cartilage proteoglycan core protein deduced from cDNA clones. *J Biol Chem* 262:17757-67, 1987.

30. Doherty M, ed. Color Atlas and Text of Osteoarthritis. London: Times mirror International Publishers Limited; 1994.

31. Dudhia J, Davidson CM, Wells TM, Vynios DH, Hardingham TE, Bayliss MT: Age-related changes in the content of the C-terminal region of aggrecan in human articular cartilage. *Biochem J* 313:933-40, 1996.

32. Eyre DR: Collagens and cartilage matrix homeostasis. *Clin Orthop Relat Res*:S118-22, 2004.

33. Eyre DR, Dickson IR, van Ness K: The collagens of articular cartilage. *Sem Arthritis Rheum* 21,S2:2-11, 1991.

34. Eyre DR, Koob TJ, Van Ness KP: Quantitation of hydroxypyridinium crosslinks in collagen by high-performance liquid chromatography. *Anal Biochem* 137:380-8, 1984.

35. Eyre DR, Paz MA, Gallop PM: Cross-linking in collagen and elastin. *Ann Rev Biochem* 53:717-48, 1984.

36. Fujimoto D, Moriguchi T: Pyridinoline, a non-reducible crosslink of collagen. Quantitative determination, distribution, and isolation of a crosslinked peptide. *J Biochem (Tokyo)* 83:863-7, 1978.

37. Gibson GJ, Verner JJ, Nelson FR, Lin DL: Degradation of the cartilage collagen matrix associated with changes in chondrocytes in osteoarthrosis. Assessment by loss of background fluroescence and immunodetection of matrix components. *J Orthop Res* 19:33-42, 2001.

38. Goodfellow JW, Bullough PG: The pattern of ageing of the articular cartilage of the elbow joint. *J Bone Joint Surg Br* 49:175-81, 1967.

39. Hacker SA, Healey RM, Yoshioka M, Coutts RD: A methodology for the quantitative assessment of articular cartilage histomorphometry. *Osteoarthritis Cartilage* 5:343-55, 1997.

40. Hall AC, Urban JPG, Gehl KA: The effects of hydrostatic pressure on matrix synthesis in articular cartilage. *J Orthop Res* 9:1-10, 1991.

41. Heinegard D, Oldberg A: Structure and biology of cartilage and bone matrix noncollageneous macromolecules. *FASEB J* 3:2042-51, 1989.

42. Heinegard D, Sommarin Y: Proteoglycans: an overview. *Methods Enzymol* 144:305-19, 1987.

43. Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, Poole AR: Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. *J Clin Invest* 93:1722-32, 1994.

44. Hollander AP, Pidoux I, Reiner A, Rorabeck C, Bourne R, Poole AR: Damage to type II collagen in aging and osteoarthritis starts at the articular surface, orginates around chondrocytes, and extends into the cartilage with progressive degeneration. *J Clin Invest* 96:2859-69, 1995.

45. Hoyland JA, Thomas JT, Donn R, Marriott A, Ayad S, Boot-Handford RP, Grant ME, Freemont AJ: Distribution of type X collagen mRNA in normal and osteoarthritic human cartilage. *Bone Miner* 15:151-63, 1991.

46. Hunziker EB: Articular cartilage structure in humans and experimental animals. In: *Articular Cartilage and Osteoarthritis,* ed. by KE Kuettner, Schleyerbach R, Peyron JG, Hascall VC, Raven Press, New York, 1992, 183-99.

47. Hunziker EB, Quinn TM, Hauselmann HJ: Quantitative structural organization of normal adult human articular cartilage. *Osteoarthritis Cartilage* 10:564-72, 2002.

48. Irwin MH, Silvers SH, Mayne R: Monoclonal antibody against chicken type IX collagen: preparation, characterization, and recognition of the intact form of type IX collagen secreted by chondrocytes. *J Cell Biol* 101:814-23, 1985.

49. Jasin HE, Dingle JT: Human mononuclear cell factors mediate cartilage matrix degradation through chondrocyte activation. *J Clin Invest* 68:571-81, 1981.

50. Kempson GE: Relationship between the tensile properties of articular cartilage from the human knee and age. *Ann Rheum Dis* 41:508-11, 1982.

51. Kempson GE: Age-related changes in the tensile properties of human articular cartilage: a comparative study between the femoral head of the hip joint and the talus of the ankle joint. *Biochim Biophys Acta* 1075:223-30, 1991.

52. Kempson GE, Muir H, Pollard C, Tuke M: The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. *Biochim Biophys Acta* 297:456-72, 1973.

53. Lohmander LS, Neame PJ, Sandy JD: The structure of aggrecan fragments in human synovial fluid. *Arthritis Rheum* 36:1214-22, 1993.

54. Mankin HJ, Dorfman H, Lipiello L, Zarins A: Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. *J Bone Joint Surg Am* 53-A:523-37, 1971.

55. Maroudas A: Balance between swelling pressure and collagen tension in normal and degenerate cartilage. *Nature* 260:808-9, 1976.

56. Maroudas A: Physico-chemical properties of articular cartilage. In: *Adult Articular Cartilage*, ed. by MAR Freeman, Pitman Medical, Tunbridge Wells, England, 1979, 215-90.

57. Maroudas A, Bayliss MT, Venn MF: Further studies on the composition of human femoral head cartilage. *Ann Rheum Dis* 39:514-23, 1980.

58. Maroudas A, Venn M: Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. II. Swelling. *Ann Rheum Dis* 36:399-406, 1977.

59. Maroudas A, Wachtel E, Grushko G, Katz EP, Weinberg P: The effect of osmotic and mechanical pressures on water partitioning in articular cartilage. *Biochim Biophys Acta* 1073:285-94, 1991.

60. Maroudas A, Ziv I, Weisman N, Venn M: Studies of hydration and swelling pressure in normal and osteoarthritic cartilage. *Biorheology* 22:159-69, 1985.

61. Martel-Pelletier J, Welsch DJ, Pelletier JP: Metalloproteases and inhibitors in arthritic diseases. *Best Pract Res Clin Rheumatol* 15:805-29, 2001.

62. McGowan KB, Kurtis MS, Lottman LM, Watson D, Sah RL: Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen® and Hoechst 33258. *Osteoarthritis Cartilage* 10:580-7, 2002.

63. Meachim G: Light microscopy of Indian ink preparations of fibrillated cartilage. *Ann Rheum Dis* 31:457-64, 1972.

64. Meachim G, Emery IH: Quantitative aspects of patello-femoral cartilage fibrillation in Liverpool necropsies. *Ann Rheum Dis* 33:39-47, 1974.

65. Meachim G, Sheffield SR: Surface ultrastructure of mature adult human articular cartilage. *J Bone Joint Surg Br* 51-B:529-39, 1969.

66. Meachim G, Stockwell RA: The matrix. In: *Adult Articular Cartilage,* ed. by MAR Freeman, Pitman Medical, Tunbridge Wells, England, 1979, 1-66.

67. Melching LI, Roughley PJ: The role of link protein in mediating the interaction between hyaluronic acid and newly secreted proteoglycan subunits from adult human articular cartilage. *J Biol Chem* 260:16279-85, 1985.

68. Mendler M, Eich-Bender SG, Vaughan L, Winterhalter KH, Bruckner P: Cartilage contains mixed fibrils of collagen types II, IX, and XI. *J Cell Biol* 108:191-8, 1989.

69. Meuller-Glauser W, Humbel B, Glatt M, Streauli P, Winterhalter KH, Bruckner P: On the role of type IX collagen in the extracellular matrix of cartilage: type IX collagen is localized to intersections of collagen fibrils. *J Cell Biol* 102:1931-9, 1986.

70. Mitrovic D, Quintero M, Stankovic A, Ryckewaert A: Cell density of adult human femoral condylar articular cartilage. *Lab Invest* 49:309-16, 1983.

71. Mizel SB, Dayer JM, Krane SM, Mergenhagen SE: Stimulation of rheumatoid synovial cell collagenase and prostaglandin production by partially purified lymphocyte-activating factor (interleukin 1). *Proc Natl Acad Sci U S A* 78:2474-7, 1981.

72. Mohtai M, Gupta MK, Donlon B, Ellison B, Cooke J, Gibbons G, Schurman DJ, Smith RL: Expression of interleukin-6 in osteoarthritic chondrocytes and effects of fluid-induced shear on this expression in normal human chondrocytes *in vitro*. *J Orthop Res* 14:67-73, 1996.

73. Morris NP, Beachinger HP: Type XI collagen is a heterotrimer with the composition 1 alpha, 2 alpha, 3 alpha retaining non-triple-helical domains. *J Biol Chem* 262:11345-50, 1987.

74. Morrison JB: The mechanics of the knee joint in relation to normal walking. *J Biomech* 3:51-61, 1970.

75. Mow VC, Zhu W, Ratcliffe A: Structure and function of articular cartilage and meniscus. In: *Basic Orthopaedic Biomechanics,* ed. by VC Mow, Hayes WC, Raven Press, New York, 1991, 143-98.

76. Muir IHM: Articular Cartilage: Biochemistry. In: *Adult Articular Cartilage,* ed. by MAR Freeman, Pitman Medical, Tunbridge Wells, England, 1979, 145-214.

77. Niyibizi C, Sagarrigo Visconti C, Gibson G, Kavalkovich K: Identification and immunolocalization of type X collagen at the ligament-bone interface. *Biochem Biophys Res Commun* 222:584-9, 1996.

78. Noyes FR, Stabler CL: A system for grading articular cartilage lesions at arthroscopy. *Am J Sports Med* 17:505-13, 1989.

79. Oldberg A, Antonsson P, Hedbom E, Heinegard D: Structure and function of extracellular matrix proteoglycans. *Biochem Soc Trans* 18:789-92, 1990.

80. Oldberg A, Antonsson P, Lindblom K, Heinegard D: A collagen-binding 59-kd protein (fibromodulin) is structurally related to the small interstitial proteoglycans PG-S1 and PG-S2 (decorin). *EMBO J* 8:2601-4, 1989.

81. Outerbridge RE: The etiology of chondromalacia patellae. *J Bone Joint Surg Br* 43-B:752-7, 1961.

82. Pokharna HK, Monnier V, Boja B, Moskowitz RW: Lysyl oxidase and maillard reaction-mediated crosslinks in aging and osteoarthritic rabbit cartilage. *J Orthop Res* 13:13-21, 1995.

83. Poole AR: Imbalances of anabolism and catabolism of cartilage matrix components in osteoarthritis. In: *Osteoarthritic Disorders,* ed. by KE Kuettner, Goldberg VM, American Academy of Orthopaedic Surgeons, Rosemont, IL, 1995, 247-60.

84. Poole AR, Pidoux I, Reiner A, Tang L-H, Choi N, Rosenberg L: Localization of proteoglycan monomer and link protein in the matrix of bovine articular cartilage: an immunohistochemical study. *J Histochem Cytochem* 28:621-35, 1980.

85. Poole CA, Ayad S, Schofield JR: Chondrons from articular cartilage: I. Immunolocalization of type VI collagen in the pericellular capsule of isolated canine tibial chondrons. *J Cell Sci* 90:635-43, 1988.

86. Prockop DJ, Kivirikko KI: Hydroxyproline and the metabolism of collagen. Biology of Collagen. In: by BS Gould, Academic Press, Indianapolis, IN, 1968, 215-46.

87. Quintero M, Mitrovic DR, Stankovic A, de Seze S, Miravet L, Ryckewaert A: Aspects cellulaires du vieillissement du cartilage articulaire. I. cartilage condylien a surface normale, preleve dans les genoux normaux. *Revue du Rhumatisme* 51:375-9, 1984.

88. Quintero M, Mitrovic DR, Stankovic A, de Seze S, Miravet L, Ryckewaert A: Aspects cellulaires du vieillissement du cartilage articulaire. II. cartilage condylien a surface fissuree preleve dans les genoux normaux et arthrosiques. *Revue du Rhumatisme* 51:445-9, 1984.

89. Ratcliffe A, Mow VC: Articular Cartilage. In: *Extracellular Matrix,* ed. by WD Comper, Overseas Publishers Association, Amsterdam, 1996, 143-98.

90. Reiser K, McCormick RJ, Rucker RB: Enzymatic and nonenzymatic cross-linking of collagen and elastin. *FASEB J* 6:2439-49, 1992.

91. Saito M, Marumo K, Fujii K, Ishioka N: Single-column high-performance liquid chromatographic-fluorescence detection of immature, mature, and senescent cross-links of collagen. *Anal Biochem* 253:26-32, 1997.

92. Sandy J, Verscharen C: Analysis of aggrecan in human knee cartilage and synovial fluid indicates that aggrecanase (ADAMTS) activity is responsible for the catabolic turnover of whole aggrecan whereas MMP-like activity is required primarily for C-terminal processing of the molecule. *Biochem J* 358:615-26, 2001.

93. Sandy JD, Neame PJ, Boynton RE, Flannery CR: Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. *J Biol Chem* 266:8683-5, 1991.

94. Schenk RK, Eggli PS, Hunziker EB: Articular cartilage morphology. In: *Articular Cartilage Biochemistry,* ed. by K Kuettner, Schleyerbach R, Hascall VC, Raven Press, New York, 1986, 3-22.

95. Schinagl RM, Gurskis D, Chen AC, Sah RL: Depth-dependent confined compression modulus of full-thickness bovine articular cartilage. *J Orthop Res* 15:499-506, 1997.

96. Schumacher BL, Su J-L, Lindley KM, Kuettner KE, Cole AA: Horizontally oriented clusters of multiple chondrons in the superficial zone of ankle, but not knee articular cartilage. *Anat Rec* 266:241-8, 2002.

97. Shapiro F, Glimcher MJ: Induction of osteoarthrosis in the rabbit knee joint. Histologic changes following menisectomy and meniscal lesions. 147:287-95, 1980.

98. Silver FH, Bradica G, Tria A: Elastic energy storage in human articular cartilage: estimation of the elastic modulus for type II collagen and changes associated with osteoarthritis. *Matrix Biol* 21:129-37, 2002.

99. Smith RL, Trindade MC, Ikenoue T, Mohtai M, Das P, Carter DR, Goodman SB, Schurman DJ: Effects of shear stress on articular chondrocyte metabolism. *Biorheology* 37:95-107, 2000.

100. Stockwell RA: The cell density of human articular and costal cartilage. *J Anat* 101:753-63, 1967.

101. Swanson SAV: Friction, Wear, and Lubrication. In: *Adult Articular Cartilage,* ed. by MAR Freeman, Pitman Medical, Tunbridge Wells, England, 1979, 415-60.

102. Takahashi M, Hoshino H, Kushida K, Inoue T: Direct measurement of crosslinks, pyridinoline, deoxypyridinoline, and pentosidine, in the hydrolysate

of tissues using high-performance liquid chromatography. *Anal Biochem* 232:158-62, 1995.

103. Uchiyama A, Ohishi T, Takahashi M, Kushida K, Inoue T, Fujie M, Horiuchi K: Fluorophores from aging human articular cartilage. *J Biochem Tokyo* 110:714-8, 1991.

104. Urban JP, Maroudas A, Bayliss MT, Dillon J: Swelling of proteoglycans at the concentrations found in cartilaginous tissues. *Biorheology* 16:447-64, 1979.

105. Vaughan L, Mendler M, Huber S, Bruckner P, Winterhalter KH, Irwin MH, Mayne R: D-periodic distribution of collagen type IX along cartilage fibrils. *J Cell Biol* 106:991-7, 1988.

106. Verzijl N, DeGroot J, Ben ZC, Braun-Benjamin O, Maroudas A, Bank RA, Mizrahi J, Schalkwijk CG, Thorpe SR, Baynes JW, Bijlsma JW, Lafeber FP, TeKoppele JM: Crosslinking by advanced glycation end products increases the stiffness of the collagen network in human articular cartilage: a possible mechanism through which age is a risk factor for osteoarthritis. *Arthritis Rheum* 46:114-23, 2002.

107. von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Glückert K, Stöss H: Type X collagen synthesis in human osteoarthritic cartilage. *Arthritis Rheum* 35:806-10, 1992.

108. Weiss C, Rosenberg L, Helfet AJ: An ultrastructural study of normal young adult human articular cartilage. *J Bone Joint Surg Am* 50-A:663-74, 1968.

109. Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 93:440-7, 1961.

110. Wong M, Wuethrich P, Eggli P, Hunziker E: Zone-specific cell biosynthetic activity in mature bovine articular cartilage: a new method using confocal microscopic stereology and quantitative autoradiography. *J Orthop Res* 14:424-32, 1996.

111. Yamada K, Healey R, Amiel D, Lotz M, Coutts R: Subchondral bone of the human knee joint in aging and osteoarthritis. *Osteoarthritis Cartilage* 10:360-9, 2002.

# CHAPTER 2

# AGE- AND SITE-ASSOCIATED BIOMECHANICAL WEAKENING OF HUMAN ARTICULAR CARTILAGE OF THE FEMORAL CONDYLE

## 2.1 Abstract

Objective. To determine the time sequence of biochemical and structural events associated with, and hypothesized to underlie, age-associated tensile weakening of macroscopically normal adult human articular cartilage of the knee.

Methods. Macroscopically normal human articular cartilage of the lateral and medial femoral condyles from Young (21-39 yrs), Middle (40-59 yrs), and Old (≥60 yrs) age donors were analyzed for tensile properties, surface wear, and cell and matrix composition.

Results. Variations in these properties were indicative of early, intermediate, and late stages of age-associated cartilage deterioration, occurring at an earlier age in the medial than the lateral femoral condyle. Early-intermediate stage changes included decreased mechanical function in the superficial zone, with a loss of (or low) tensile integrity, and surface wear, with faint striations and mild staining on the articular surface after application of India ink. Intermediate-late stage changes included

maintenance of moderate level biomechanical function, a decrease in cellularity, and a decrease in matrix glycosaminoglycan content. Tissue fluorescence increased steadily with age.

Conclusions. Many of these age-associated differences are identical to those regarded as pathological features of cartilage degeneration in early osteoarthritis. These findings provide evidence for the roles of mechanical wear and cell death, and enzymatic degradation in mediating the progression through successive and distinguishable stages of early cartilage deterioration.

## 2.2 Introduction

With aging, the tensile properties of macroscopically normal adult human knee articular cartilage diminish markedly [25]. In particular, tensile strength and stiffness in the superficial layer of human femoral condylar cartilage diminish by ~65% from peak values at ~24 years of age to values at ~90 yrs of age. In the deeper layers of cartilage, tensile strength also decreases with age in the adult, with strength values being ~50% of those of superficial layer cartilage at the same ages. Such age-related weakening of cartilage may predispose the knee joint to development of pain, dysfunction, and the classical histopathological features of osteoarthritis (OA). However, it is unknown how the age-related biomechanical changes vary between sites within the knee joint and what the structural or compositional bases for these changes are.

Biomechanical weakening of articular cartilage with age may be due to alterations in cellularity or matrix properties, and reflect 1) consequences of cell death, 2) mechanical wear at the surface, 3) degradation of extracellular matrix components, or 4) modification of the collagen network through crosslinks. Mechanical weakening with age may arise from cell death and reflect an inability of the remaining cells to synthesize and remodel matrix components to maintain tissue homeostasis. However, whether or not cartilage undergoes an age-associated decrease in cellularity is controversial. The cell density, as determined from cell counting in histological sections, of macroscopically normal human articular cartilage of the femoral condyles has been shown to decrease with age [33, 43], but may be dependent on anatomical

location and depth from the articular surface [49]. Further, it is unclear if this decrease in cellularity is due to cell death or if the presence of empty lacunae is due to cell loss during histological preparation [1]. Alternatively, cartilage weakening may relate directly to mechanical wear [31], with both cartilage weakening and mechanical wear being a manifestation of disruption of the collagen network, especially at or near the articular surface [12]. In addition, mechanical weakening may be due to degradation of extracellular matrix components. Chondrocyte and synoviocyte production of pro-inflammatory cytokines and proteases [8, 13] may cause cleavage and denaturation of the collagen network [5, 22], fragmentation and loss of proteoglycans [14, 45], and tissue weakening. However, these may be manifestations of more advanced stages of osteoarthritic degeneration. Finally, modifications to the collagen network such as the accumulation with age of non-enzymatic glycation products, including pentosidine [4, 51], have been associated with a decrease in instantaneous deformation and increased collagen stiffening [4]. This could give rise to a more brittle and fragile collagen network and contribute to age-associated tensile weakening [10]. These four postulated mechanisms of biomechanical weakening with age may, alone or in combination, underlie progressive cartilage degeneration and, ultimately, end-stage OA.

The site-specific pattern and severity of changes in cartilage properties in the knee joint may give insight into the mechanism of age-related cartilage weakening. The compositional, structural, and functional properties of articular cartilage appear to be modulated by the extent and pattern of joint loading across the knee joint. Areas of high weight-bearing are associated with a relatively high concentration of aggrecanase

cleavage products [6] and low equilibrium tensile modulus [2] compared to areas loaded more intermittently. In addition, the medial compartment of the knee is subjected to higher joint pressures than the lateral compartment during the stance phase of normal gait [18, 34], a loading pattern that may cause early degenerative changes to be more prevalent in the medial femoral condyle (MFC) than the lateral femoral condyle (LFC) [7]. When degenerative changes are present, they are usually more severe in the MFC than the LFC [35] and include a decrease in cartilage thickness of the MFC, but not the LFC of patients with knee OA [9]. Functionally, the cartilage of the MFC also has a lower indentation stiffness than does that of the LFC [3, 28]. Cartilage function, composition, and structure appear to change progressively in early degeneration and OA [2, 22, 39, 44]. Thus, comparison of the MFC and LFC cartilage with respect to age-associated differences in the function, composition, and structure may provide insight into the mechanisms of age- and OA disease-associated degeneration.

The hypothesis underlying this study was that human articular cartilage, devoid of gross erosion, exhibits tensile softening with age in a site-associated manner due to changes in composition and structure, reflecting sequential stages of early cartilage degeneration. As a first step to test a causal link between specific changes and biomechanical weakening, this study sought to establish the extent of variations in the relevant properties of individual human tissue samples that were macroscopically normal with age. Once principal age-associated changes are established, further study with experimentally-controlled manipulations of those properties could test the role of specific processes as a pathogenic mechanism resulting in cartilage degeneration.

Thus, the objectives were to analyze, in macroscopically normal and structurally characterized human articular cartilage from adults of young, middle, and old age and taken from different depths at the LFC and MFC sites, (1) tensile biomechanical properties, (2) density of cells, (3) content of extracellular matrix components, and (4) fluorescence indicative of non-enzymatic glycation products. The results were interpreted in terms of the mechanisms of cartilage weakening, postulated above, and early, intermediate, and late adult stages of age-associated degeneration.

## 2.3 Materials and Methods

### 2.3.1. Sample Selection and Preparation

Samples were selected from the anterior region of the MFC ($n$=28) and LFC ($n$=28) from 31 human cadaveric donors distributed among three adult age groups, Young (20-39 yrs), Middle (40-59 yrs), and Old (≥60 yrs). Samples were obtained from tissue banks with donation areas in the Western and Southern areas of the United States. The samples were from donors that were distributed approximately evenly between males ($n$=4-7) and females ($n$=3-5) within each age group and that had a similar distribution of body mass index (BMI) between age groups (Table 2.1).

| | | Lateral Femoral Condyle | | | Medial Femoral Condyle | | |
|---|---|---|---|---|---|---|---|
| | | Young | Middle | Old | Young | Middle | Old |
| Age (yr) | Female | 33±2 | 47±3 | 73±5 | 33±2 | 47±3 | 70±2 |
| | Male | 29±3 | 49±1 | 69±2 | 29±3 | 49±1 | 70±3 |
| | Female+Male | 30±2 | 48±1 | 70±3 | 30±2 | 48±1 | 70±2 |
| BMI ($kg/m^2$) | Female | 28±4 | 19±1 | 24±3 | 28±4 | 19±1 | 19±3 |
| | Male | 25±1 | 24±2 | 24±1 | 25±1 | 24±2 | 24±1 |
| | Female+Male | 26±2 | 23±1 | 24±2 | 26±2 | 23±1 | 22±2 |
| *n* | Female | 5 | 3 | 5 | 5 | 3 | 4 |
| | Male | 7 | 7 | 6 | 7 | 7 | 5 |
| | Female+Male | 12 | 10 | 11 | 12 | 10 | 9 |

**Table 2.1:** Donor description. Age, body mass index (BMI), and *n* of human female and male donors of osteochondral cores from the LFC and MFC reported as mean ± SEM.

Samples used for this study were the normal subsets of samples used, in part, in a previous study on indentation stiffness and structural indices of wear [3]. These samples were considered normal because, at harvest, they were devoid of macroscopic cartilage erosion and from joints without full-thickness erosion to bone in the femoral compartment. In the prior study, parts of these samples were examined for cartilage thickness, histopathology (Mankin-Shapiro score, surface irregularity), and surface roughening (reflectance score after India ink staining). Cartilage thickness (Figure 2.1a) did not vary with age group and did not vary with medial or lateral locations, consistent with the selection of non-eroded cartilage specimens. The reflectance score decreased with age group, indicating more ink staining, and was lower in MFC samples compared to those of the LFC (Figure 2.1b). In addition, the variance of the reflectance score indicated more variation in ink-staining with age in the LFC and high variation in all age groups of the MFC (Figure 2.1c). Consistent with this, the Mankin-Shapiro histopathology score [47] of these normal samples, while being low in general (e.g., compared to degenerate samples), increased slightly in the MFC from Young to Old age groups (Figure 2.1d), primarily due to a higher surface irregularity (Figure 2.1e). Thus, while these samples were judged to be macroscopically normal, there was evidence for very mild age- and site-associated surface roughening at the articular surface.

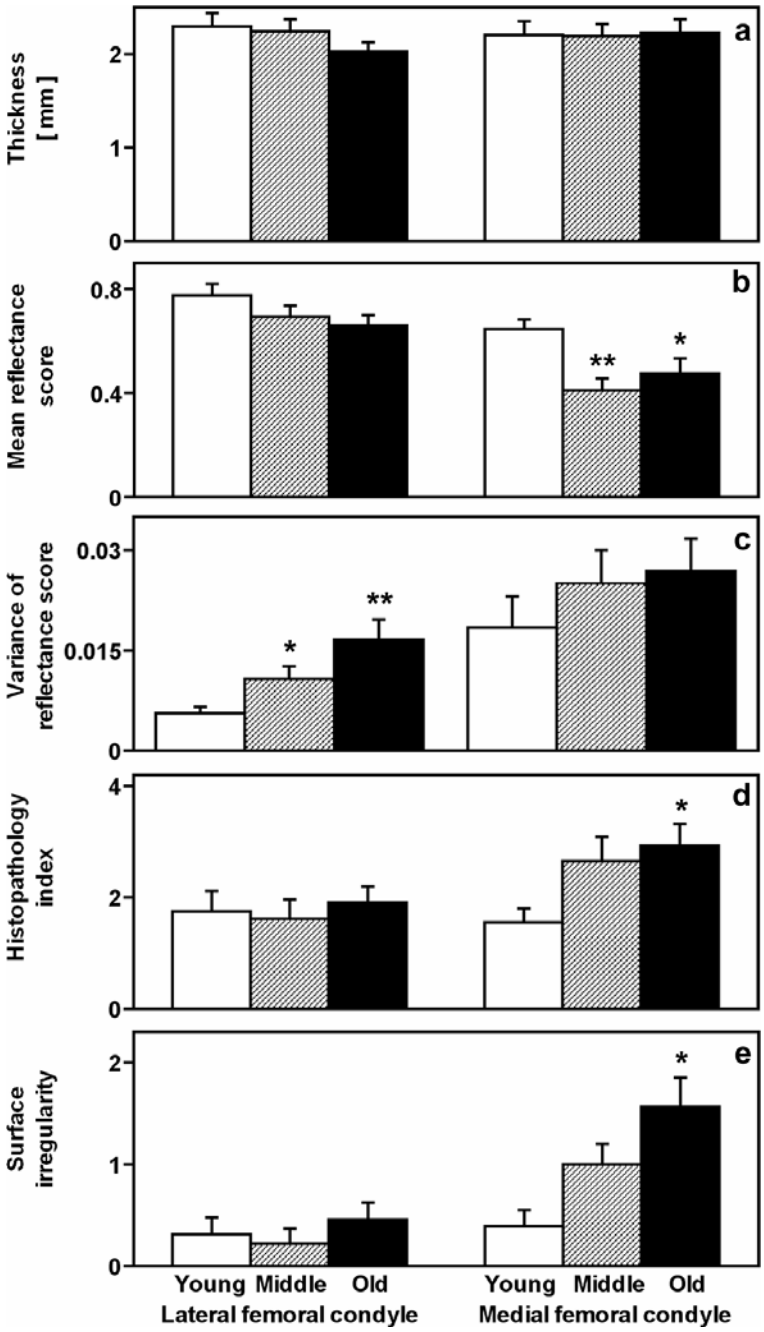



**Figure 2.1:** Structural and surface properties of human articular cartilage from the LFC and MFC. Cartilage thickness (**a**), reflectance score assessed after India ink staining (**b**), the variance of reflectance score (**c**), overall histopathological index of cartilage degeneration (**d**), and surface irregularity assessed by histopathological grading (**e**) from donors of Young (21-39 yrs old), Middle (40-59 yrs old), and Old (≥60 yrs old) age groups. *n*=8-12. *p<0.05, **p<0.005 versus Young age samples.

These samples were initially harvested in the form of 10-mm diameter osteochondral cores with a notch placed in the posterior-most edge of the core in order to maintain orientation. Samples were soaked in an excess volume (~5 ml each) of phosphate buffered saline (PBS) with proteinase inhibitors (PI; 1 mM PMSF, 2 mM EDTA, 5 mM benzamidine hydrochloride, and 10 mM N-ethylmaleimide) [16] at 4°C for one hour and then stored at –70°C until use. Samples were thawed in ~1 ml of PBS with PI for 15 minutes at room temperature prior to analysis. Control studies with cartilage slices from Young and Old donors confirmed that there was negligible loss of GAG or DNA into the PBS+PI bath solution (<3% of the total).

### 2.3.2. Biomechanical Properties

Portions of each core were separated into superficial, middle, and deep layers and analyzed by tensile testing. The cartilage of each core was sliced into ~0.3-mm thick layers, at a distance from the articular surface of 0% (superficial layer, including the articular surface), 30% (middle layer), and 60% (deep layer) of the average cartilage thickness. These slices were cut into tapered specimens, with a 0.8-mm wide and 4-mm long gage region oriented parallel to the splitline pattern, for tensile testing using a methodology [54] that is a combination of previous equilibrium and constant strain-rate test protocols [2, 26]. The force data were normalized to the width and thickness of the sample to obtain stress in units of megapascals (MPa), and the displacement data were normalized to the initial length to obtain strain (dimensionless). The equilibrium test results were analyzed to determine tensile equilibrium modulus, and the dynamic test results were used to assess tensile ramp modulus, tensile strength, and failure strain.

### 2.3.3. Biochemical Properties

Portions of tissue slices, adjacent to tensile samples, were analyzed for cell and matrix properties. These portions were weighed wet, lyophilized, weighed dry, and solubilized with proteinase K (ProK). These solubilized portions were used to determine total DNA [30], hydroxyproline [55], and glycosaminoglycan (GAG) content [15], as well as intrinsic fluorescence at excitation (Ex) and emission (Em) wavelengths corresponding to maximum fluorescence of pyridinoline (Ex 295/Em 395 nm [17]) and pentosidine (Ex 335/Em 385 nm [46]) crosslinks, consistent with peaks in fluorescence maps [30]. DNA was converted to cell number using a conversion factor of 7.3 pg DNA/human chondrocyte [49]. Hydroxyproline content was converted to collagen (COL) content using a mass ratio of collagen to hydroxyproline equal to 7.1 [24]. Sulfated GAG content was calculated by comparison to known concentrations of shark chondroitin sulfate. The contents of DNA, COL, and GAG were calculated as the mass normalized to wet weight. Fluorescence data are reported as a ratio of pentosidine-associated fluorescence to pyridinoline-associated fluorescence, the latter of which are stable with age [4].

The residual portions of the cartilage sections were analyzed for denatured collagen exactly as described previously [5]. Briefly, proteoglycan was extracted with guanidine hydrochloride (Gnd), degraded collagen was extracted with alpha-chymotrypsin (aCT), and the remaining tissue was digested with ProK. As a positive control for detecting denatured collagen, this analysis was also performed on portions of full-thickness cartilage from the patellae of a 56 yr old human donor, with some portions analyzed directly and other portions heated to 80°C for 4 hours in 0.1 M

sodium phosphate and PI to denature the collagen in a controlled manner. The aCT and ProK solutions were analyzed for COL as described above, and the percent of COL in aCT (denatured) was calculated as that in aCT compared to the sum in aCT and ProK solutions.

### 2.3.4. Statistics

The effect of age group on the various mechanical and biochemical parameters was assessed using repeated measures ANOVA with anatomical location (LFC or MFC) and depth from the surface (superficial, middle, or deep) as repeated factors. When anatomical location was found to have a significant effect ($p<0.05$), the locations were analyzed separately. When age group or depth from the articular surface had an effect (for $p<0.05$), planned comparisons were made between age groups at each depth or between depths for each age group. To limit the experimentwise error rate, each comparison was tested using a significance level alpha = 0.05 divided by the number of comparisons made [48]. For results that were expressed as a ratio or percentage, data were arcsine transformed to improve normality, prior to the above statistical analyses. All data are reported as mean ± SEM.

## 2.4 Results

**Biomechanical Properties.** Variation of tensile properties with age group occurred in a manner that was dependent on sample anatomical location (site) and depth from the articular surface (Figure 2.2). Ramp modulus (Figure 2.2b), strength (Figure 2.2c), and failure strain (Figure 2.2d) were lower in the MFC than LFC ($p<0.005$, $p<0.01$, and

$p<0.005$, respectively), and equilibrium modulus (Figure 2.2a) showed a similar trend ($p=0.2$). Each of these tensile properties was depth-dependent (each, $p<0.005$), with the equilibrium modulus being 167% higher, the ramp modulus being 176% higher, the strength being 29% higher, and the failure strain being 54% lower in the superficial layer than the deep layer. More specific age-associated differences are described below, as a function of site and depth.

In the LFC, the tensile integrity of superficial, middle, and deep layers varied with age in a manner suggesting that the superficial layer was affected earliest (by Middle age), while the middle layer was affected later (by Old age), with the deep layer not showing any age-related variation. In the LFC, the tensile integrity of the superficial layer of the Young age group was higher than that of the Middle and Old age groups. The equilibrium modulus (Figure 2.2a), ramp modulus (Figure 2.2b), and strength (Figure 2.2c) of the superficial layer were 39% ($p=0.2$) and 41% ($p=0.07$), 21% ($p<0.01$) and 30% ($p<0.005$), and 37% ($p<0.005$) and 40% ($p<0.005$) lower, respectively, in Middle and Old age samples than corresponding values in Young age samples. In middle layer cartilage of the LFC, the ramp modulus ($p=0.09$), and strength ($p=0.04$) tended to be lower in the Old age group than the Young age group. In the LFC, failure strain did not vary between age groups in superficial, middle, or deep layers ($p=0.09$-$0.8$, Figure 2.2d).

In the MFC, tensile integrity showed a pattern of variation distinctly different from that of the LFC; the MFC showed relatively little variation with age and depth. In the MFC, the tensile integrity of the superficial layer was relatively low in all age groups. In the superficial, middle, and deep layers, the equilibrium modulus ($p=0.3$-

0.9, Figure 2.2a), ramp modulus (p=0.2-1.0, Figure 2.2b) and strength (p=0.09-0.8, Figure 2.2c) did not vary with age group. The failure strain of the superficial layer was higher in Middle age samples ($p<0.025$, Figure 2.2d), but did not vary with age in the middle or deep layers (p=0.05-1.0). Taken together, these results show a pattern of age-associated tensile weakening which occurs at an early age in the LFC and evidence for a tissue that is already weak by a Young age in the MFC.

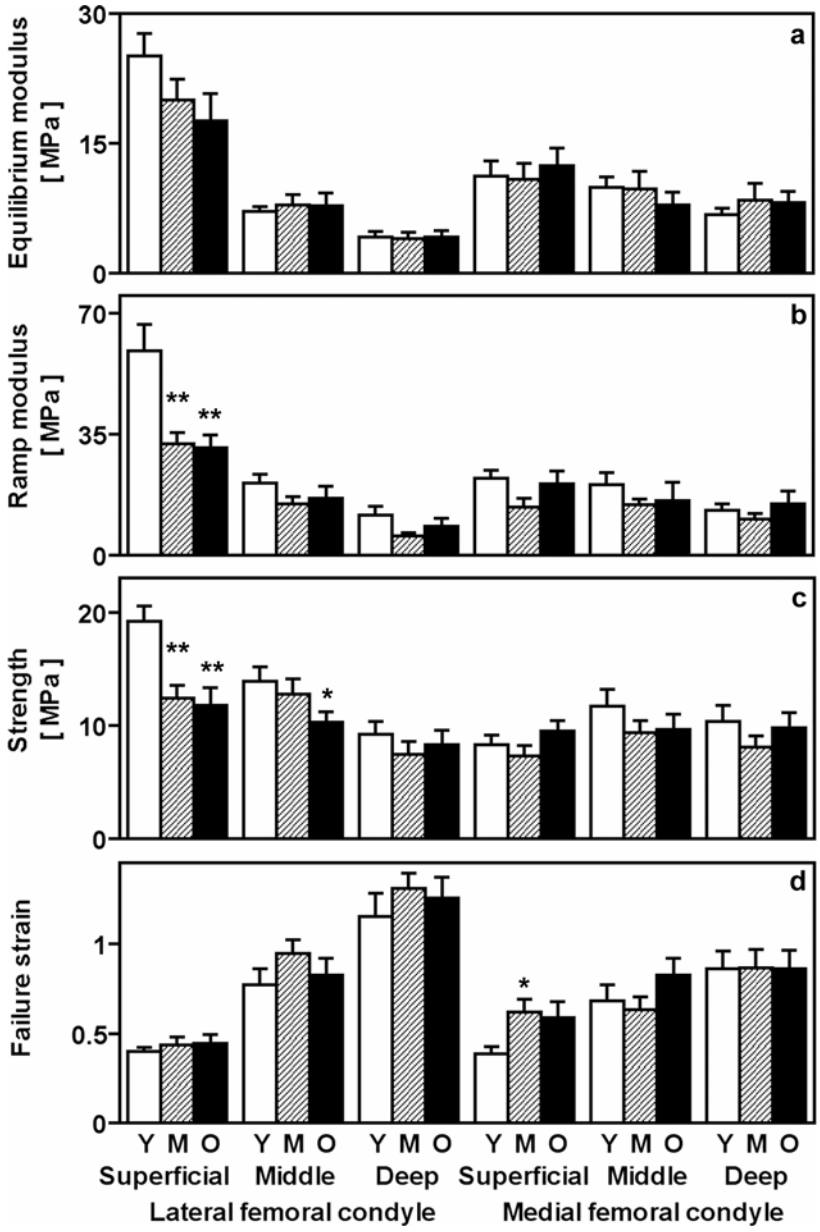



**Figure 2.2:** Tensile biomechanical properties of samples described in Figure 2.1. For specimens from the superficial, middle, and deep layers, the tensile equilibrium modulus (**a**), tensile ramp modulus (**b**), tensile strength (**c**), and failure strain (**d**) were determined from equilibrium and then non-equilibrium failure testing of articular cartilage from Young (Y), Middle (M), and Old (O) age donors. *n*=9-12. *p<0.05, **p<0.005 versus Young age samples.

**Biochemical Properties.** Variation of biochemical properties with age group also occurred in a manner that was dependent on site and depth from the surface (Figure 2.3). Water content (Figure 2.3a), DNA (Figure 2.3b), COL in aCT (Figure 2.3d), and GAG (Figure 2.3f) tended to be higher ($p<0.05$, $p=0.09$, $p<0.005$, and $p<0.05$, respectively), and COL (Figure 2.3c) and fluorescence ratio (Figure 2.3e) were lower in the MFC than the LFC, ($p<0.05$ and $p<0.005$, respectively). There was a depth-dependence in DNA ($p<0.005$), COL in aCT ($p<0.005$), fluorescence ratio ($p<0.005$), and GAG ($p<0.005$), but not water content ($p=0.2$) or COL ($p=0.5$). In particular, DNA, COL in aCT, and fluorescence ratio were 180%, 29%, and 15% higher, respectively, and GAG was 66% lower in the superficial layer than the deep layer. The controlled heating of patellar cartilage increased collagen denaturation from 8±1% to 76±3%, confirming the method of analysis of degraded collagen and the effectiveness of aCT extraction. At particular sites and depths, there were notable age-associated differences in these cartilage components, as described below. Overall, deterioration of certain biochemical properties appeared to occur at stages subsequent to the decrease of tensile integrity noted above.

The water content indicated the absence of marked aging-associated swelling of cartilage of these three age groups (Figure 2.3a). In the superficial, middle, and deep layers, the water content did not vary with age group in the LFC ($p=0.2$-$1.0$) or MFC ($p=0.3$-$1.0$) and was ~73% of the wet weight.

The DNA content of the MFC superficial layer varied with age, while that of the LFC superficial layer, and also of the middle and deep layers of the MFC and LFC did not (Figure 2.3b). In the MFC, the DNA content of the superficial layer of Young

age samples was higher than that of both Middle and Old age samples (30%, $p=0.05$ and 34%, $p=0.05$, respectively). In the LFC, the DNA content of superficial layer tended to decrease from Young to Old age (10%, $p=0.3$). These differences in DNA content occurred at a stage after tensile properties were weak (by Middle age in the MFC whereas tensile integrity was already low at a Young age, and by Old age in the LFC whereas tensile properties diminished at the Middle age). In the LFC and MFC, the DNA content of the middle and deep layers was similar between age groups ($p=0.1$-$1.0$). Thus, the pattern of age-associated decrease in DNA content of the superficial layer was delayed relative to the age-associated decrease in tensile integrity.

Age-associated changes in the collagen network were manifest as an alteration of the fluorescence ratio (Figure 2.3e), but not as an alteration of the total COL content (Figure 2.3c) or COL in aCT (Figure 2.3d). COL content and COL in aCT in the superficial, middle, and deep layers of the LFC and MFC did not show any age-associated change ($p=0.1$-$0.9$). The fluorescence ratio, on the other hand, showed an increase with age at each depth, with differences depending on the site. In the LFC superficial layer, the fluorescence ratio was higher in the Old age group than in the Young age group (by 79%, $p<0.005$), while it was higher in Middle and Old age groups in the LFC middle layer (by 51%, $p<0.025$, and by 74%, $p<0.01$, respectively), and LFC deep layer (by 26%, $p=0.09$, and by 53%, $p<0.025$, respectively). In the MFC, the fluorescence ratio in the superficial layer was higher in Middle (by 30%, $p=0.05$) and Old (by 85%, $p<0.005$) age samples than Young age samples, but was not distinguishably different with age in middle and deep layers ($p=0.08$-$0.7$). Taken

together, these results indicated that although the content of collagen and degradation products in these macroscopically normal samples were indistinguishable in aging, there was an age-associated increase in pentosidine-associated fluorescence in the superficial layers, subsequent to tensile weakening by Middle Age in the LFC and subsequent to the weak tensile state at Young age in the MFC.

The GAG content showed age-associated differences that were localized to the deep and middle layers, and not evident in the superficial layer (Figure 2.3f). In the LFC, the GAG contents of the middle and deep layers were lower in the Old age groups than in the Young age group (by 31%, $p<0.005$, and by 34%, $p=0.04$) and also lower in the Old age groups and Middle age groups (by 25%, $p=0.2$, and by 31%, $p<0.005$). In the MFC, the GAG of the middle and deep layers tended to be lower in the Old age group than in the Young age group ($p=0.2$, and $p=0.03$, respectively). The pattern of decrease in GAG content in the middle and deep layers from Middle to Old age groups appeared subsequent to the low levels of tensile integrity in the Young (LFC) or Middle (MFC) age groups.

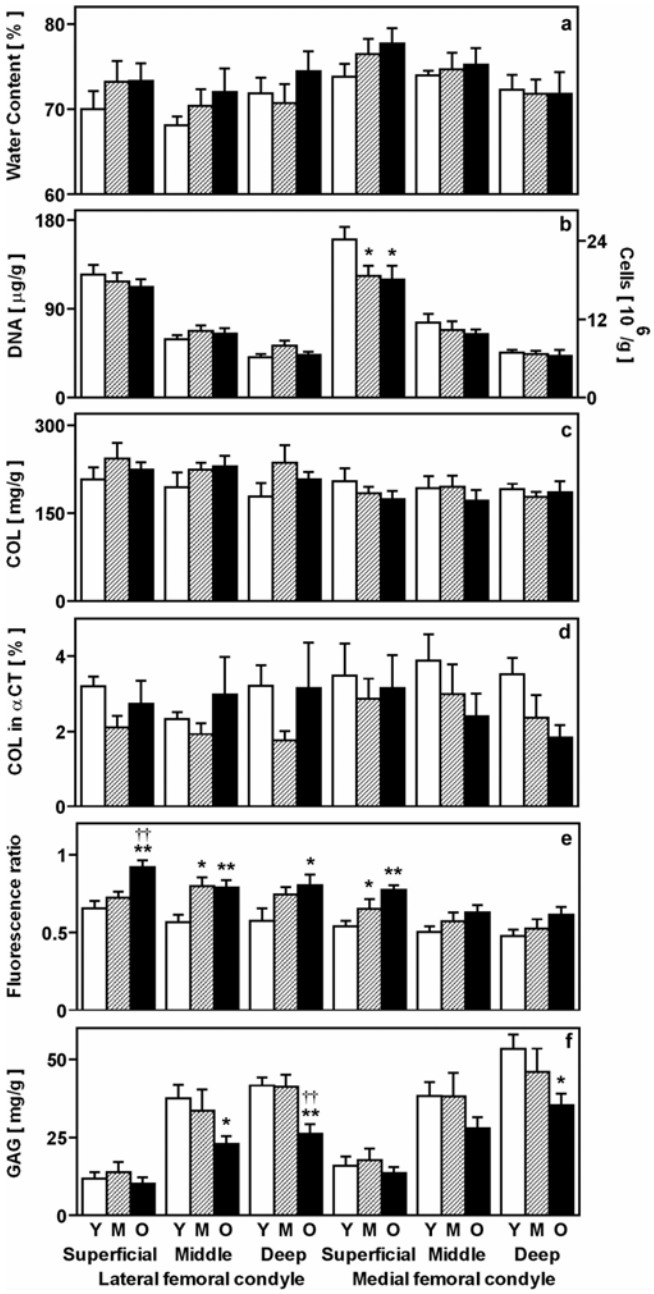



**Figure 2.3:** Biochemical properties of human articular cartilage samples described in Figure 2.1. Cartilage tissue adjacent to the mechanical test specimens was analyzed for water content (**a**), DNA and calculated cell number (**b**), COL (**c**), COL in aCT (**d**), the fluorescence ratio (**e**) of pentosidine-associated fluorescence (Ex 335/Em 385 nm) to pyridinoline-associated fluorescence (Ex 295/Em 395 nm) and GAG (**f**). DNA, COL, and GAG were each normalized to wet weight. *n*=9-12. *p<0.05, **p<0.005 versus Young age samples. ††p<0.005 versus Middle age samples.

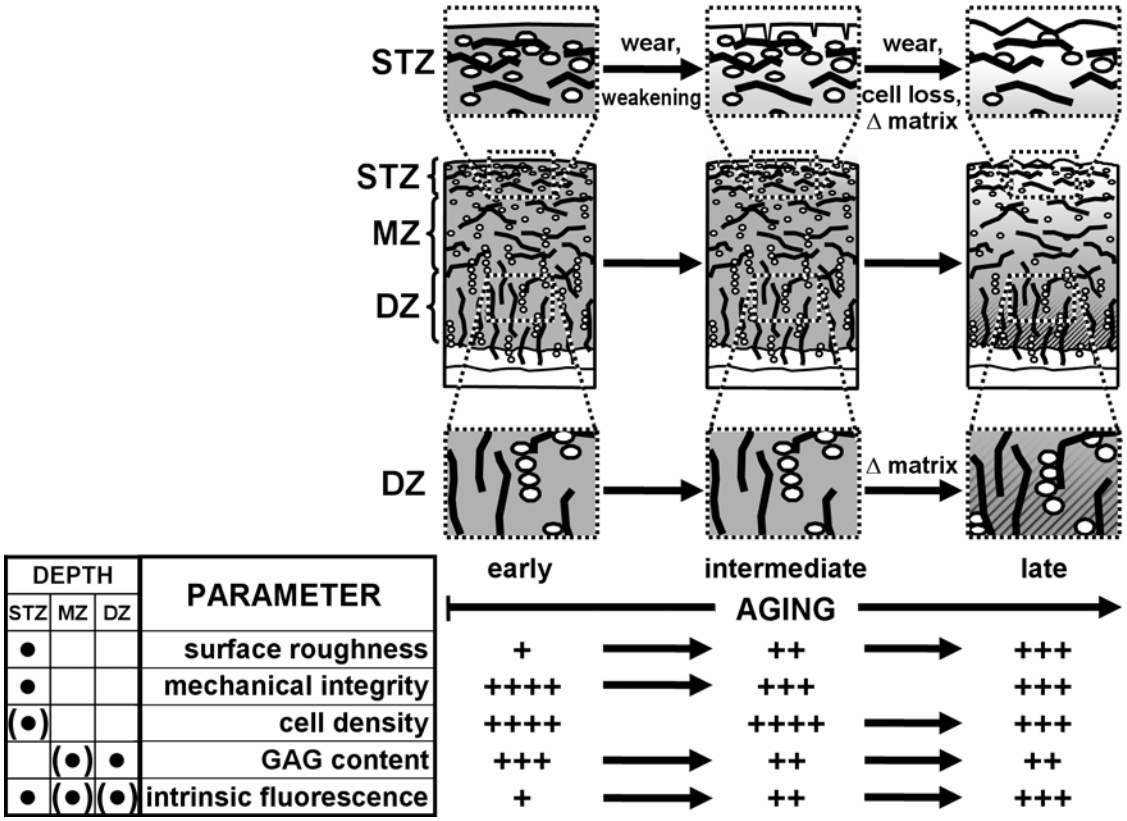
## 2.5 Discussion

This study of macroscopically normal human articular cartilage identified a pattern of age-associated changes in biomechanical integrity, cellularity, content of matrix components, and fluorescence index of non-enzymatic glycation, some of which appear related to structural indices of surface wear. These changes were prominent in cartilage at certain anatomical locations and depths. Changes of a similar sequence generally occurred at an earlier age in the MFC than in the LFC. The decrease in tensile integrity (Figure 2.2) and increase in surface wear (Figure 2.1) occurred from Young to Middle age groups in the LFC samples while low tensile integrity and surface wear were already evident in MFC samples at the Young age. This was followed by a decrease in tissue cellularity in the superficial layer (Young to Middle in the MFC, Figure 2.3b), increased fluorescence (from Middle to Old in all layers of the LFC and a trend from Middle to Old in the superficial layer of the MFC, Figure 2.3e), and decreased GAG content of the deep layer (from Middle to Old in the LFC and MFC, Figure 2.3f). These age-associated changes appear to represent stages of mild cartilage degeneration that may progress to, or predispose the joint to, the development of OA.

The changes in tensile integrity, surface wear, and tissue composition were suggestive of early, intermediate, and late stages of age-associated deterioration occurring in a zonal pattern (Figure 2.4), at an earlier age in the MFC than the LFC. Early-to-intermediate age-associated changes included surface wear and decreased mechanical function of the superficial zone, demonstrated here by India ink staining and histopathological indices of roughness as well as loss of, or low, tensile

biomechanical integrity. This was followed by intermediate-to-late stage changes, a decrease in cellularity in the superficial zone and net depletion of GAG in the deep zone. Many of these age-associated degenerative changes are essentially identical to those regarded as features of cartilage in early OA [40].

With regard to these proposed stages of age-associated articular cartilage deterioration, some differences between the MFC and LFC locations were striking. There was a greater amount of degeneration in medial than lateral samples by Old age as indicated by the increased histopathology score. Further, the reflectance scores of medial samples were much lower than those of lateral samples, especially at Middle and Old ages, indicating an increase in surface wear. Each of these would lead to the expectation for more weakened tissue in the MFC, which was the case. Features such as higher cellularity in the superficial layer and lower failure strain in middle and deep layers of MFC samples compared to those of corresponding LFC samples may indicate intrinsic differences between articular cartilage of the medial and lateral femoral condyles.




| DEPTH | | | PARAMETER | early | | intermediate | | late |
|---|---|---|---|---|---|---|---|---|
| STZ | MZ | DZ | | | | | | |
| ● | | | surface roughness | + | → | ++ | → | +++ |
| ● | | | mechanical integrity | ++++ | → | +++ | | +++ |
| (●) | | | cell density | ++++ | | ++++ | → | +++ |
| | (●) | ● | GAG content | +++ | → | ++ | → | ++ |
| ● | (●) | (●) | intrinsic fluorescence | + | → | ++ | → | +++ |

**Figure 2.4:** Summary of cartilage changes at early, intermediate, and late stages of age-associated degeneration in particular zones. Changes in superficial tangential zone (STZ), middle zone (MZ), and deep zone (DZ) are noted by ●, with parentheses signifying variable changes. Depicted are changes in mechanical integrity (degree of gray shading), articular surface wear, alterations of chondrocyte density, increase in intrinsic fluorescence, and loss of GAG (▨).

Several factors may limit the interpretation of the results of this study. Diet, lifestyle, and genetics of human donors were not controlled for or analyzed. However, samples were chosen to minimize effects of such factors by choosing approximately equal numbers of male and female donors for each age group, achieving a distribution of body mass indices that were similar between age groups, and selecting macroscopically normal, non-eroded cartilage samples. Furthermore, because normal-appearing cartilage in osteoarthritic joints have elevated levels of matrix degrading enzymes [56], the macroscopically normal samples of this study were obtained from donors without predisposing conditions for joint disorders and without full-thickness cartilage erosion in the femoral compartment. This sampling was consistent with low levels of collagen degradation [5, 22].

The analysis of human cartilage samples in discrete layers and a single orientation was done to limit sources of variability in measures of biomechanical and biochemical properties. Such an analysis of cartilage layers was done at the expense of analysis of the full thickness of tissue, although indentation analysis of adjacent tissue was described previously [3]. The standardized sampling of layers may also affect the interpretation of the results. Dimensions and orientation with respect to the splitline pattern [32] of tapered tensile test specimens were uniform among samples in this study and chosen to be similar to those of previous studies [2, 25] to allow for direct comparisons.

Further, samples including the articular surface (top of specimen at 0%) were used because of its importance to the tensile properties of articular cartilage [26] and to overall cartilage deformation during joint loading [11], and its sensitivity to aging

[25] and degeneration [2]. The age-associated increase in surface fibrillation, detected by histology and image analysis of ink-stained surfaces, could have been an indication of loss of the superficial zone of cartilage. However, the similarity of cartilage thickness among age groups (Figure 2.1a) and the histological analyses indicated that such alteration was mild, and is consistent with the selection and use of non-eroded cartilage specimens, the preservation (inclusion) of the superficial zone, and the absence of gross erosion.

The analysis of tensile integrity expanded upon results of previous studies by assessing the effect of age at each site. This allowed separation of the confounding effects of osteoarthritic degeneration, site, and age on tensile integrity, factors which typically are present because of the prevalence of OA at advanced age [41] and because cartilage degeneration occurs earlier in the medial than the lateral femoral condyle [7]. The range of values of tensile moduli and strength were generally similar to those obtained in previous studies of tensile equilibrium [2] and dynamic stiffness and failure [25]. The age-associated decrease in tensile strength of ~10% per decade of age of macroscopically normal human articular cartilage noted by Kempson [25] would appear to represent, based on the current study (Figure 2.2c), the combination of an age-associated decrease of tensile strength of the LFC and low strength of the MFC at all ages. Furthermore, the tensile weakening with age, in addition to changes with degeneration and OA, noted by Akizuki, et al. [2] (middle-age fibrillated and old-age OA cartilage), with tensile equilibrium moduli being ~85% and 70% lower than those of young normal cartilage, appears to be partially (~20%) attributable to age-associated tensile weakening demonstrated in this study (Figure 2.2a). Thus, both age

and site on the femoral condyle contribute markedly to variation in tensile cartilage biomechanical properties.

The analysis of DNA content helps to clarify the controversy about age-associated changes in cartilage cellularity at different depths. Measures of DNA in macroscopically normal human articular cartilage were made possible in small portions of tissue by an assay of DNA [30] that has improved sensitivity and specificity [52] over other assays [27, 36]. The measure of DNA in this study does indicate an age-associated decrease in cellularity, localized to the MFC superficial layer (Figure 2.3b). This result parallels the decrease of cell density as measured by cell counting in histological sections, where cell density decreased with age in macroscopically normal articular cartilage from weight-bearing zones after 40 yrs of age [33, 43] especially in the superficial zone [49]. It remains unclear if the decrease in cell density noted in the prior histological analyses is due to cell death or if the presence of empty lacunae is due to a histological artifact of cell loss [1]. The biochemical analyses support the notion that the measured DNA is within cells, rather than in the extracellular space, since tissue extracts with Gnd and aCT (that remove 90% of GAG) contained virtually no DNA (<2% of the total in the tissue, data not shown). One caveat, however, is that the biochemical measure of DNA does not describe cell activity or organization, and focal sites of hypo- and hypercellularity may exist. The decrease in cell density in the superficial zone of cartilage may contribute to the inability of remaining cells to maintain extracellular matrix in this region, and thus, to the eventual deterioration and loss of mechanical integrity.

The pattern of age-associated variation in total and denatured collagen content and the fluorescence ratio is consistent with the idea that there is some amount of collagen turnover in mature, macroscopically normal cartilage and expand upon previous studies by establishing the aging-associated sequence of changes in collagen structure. The total collagen content (Figure 2.3c) and denatured collagen content (Figure 2.3d) agreed with previously reported values [5, 22, 50] and, further, varied little with age or depth from the articular surface, agreeing with the idea that collagen denaturation may be a hallmark of early stage OA [40]. In the method used, the extractability of hydroxyproline may reflect a shift in synthesis of collagen species that have not undergone or may not undergo the level of crosslinking of type II collagen. Therefore, the changes in distribution of hydroxyproline could reflect shifts in chondrocyte metabolism with a significant alteration in the regulation of collagen expression, rather than an alteration of denaturation. While the mechanical effects of pentosidine crosslinks have been studied extensively in the past, it was, as yet, unclear if the extent of glycation that occurs with aging is sufficient to precipitate the weakening (lowered strength and ramp modulus) of cartilage seen in aging or arthritis [10]. The results of this study indicated that the increase in the fluorescence ratio (pentosidine crosslinking, Figure 2.3e) occurs at an age (from Middle to Old age in the LFC) subsequent to initial tensile weakening (from Young to Middle age in the LFC, Figure 2.2b,c). Further, in the MFC, the increase in the fluorescence ratio from Young to Old age groups was not as dramatic and may indicate the occurrence of collagen turnover at that site. It should be noted, however, that this measure of intrinsic

fluorescence was an indirect measure of crosslink fluorescence and that there may be interference from other matrix components.

The pattern of variation in GAG content with depth is generally similar to that found in previous studies [26, 29], but also showed an age-associated decrease of GAG content in the deep layer of articular cartilage (Figure 2.3f). The measure of sulfated GAG, by reaction with DMMB, was standardized to a chondroitin sulfate standard, and is indicative of charge content since keratan sulfate (one anionic charge group per disaccharide) gives half as much signal per mass as does chondroitin sulfate (two anionic charge groups per disaccharide) [15]. No attempt was made to assess specific alterations of GAG or aggrecan structure that change with skeletal development and aging [20, 21, 23, 38] and OA [37, 39]. The decrease with age of GAG in the deep layer from Mid to Old age (Figure 2.2f), occurs without additional elevation in fluorescence (Figure 2.2g), suggesting that substantial matrix turnover is occurring. The localization in the deep layer is intriguing, particularly given recent evidence that changes in subchondral bone turnover can markedly affect the overlying articular cartilage [19, 42, 53].

More work is needed to fully elucidate the causes and consequences of aging-associated tensile weakening. The results of this study point to a role for surface wear and fatigue as major changes in early cartilage deterioration with cell loss being a down-stream event. Tensile weakening may be directly related to wear at the articular surface [12, 31], with disruption of the collagen network in areas within or adjacent to striations. Consequences of this initial surface wear may include further disruption of the collagen network, loss of extracellular matrix molecules, and loss of cells. Initial

age-associated tensile weakening appears not to be due to enzyme-mediated degradation of the collagen network and proteoglycan, or to an age-associated increase in collagen crosslinking, because tensile weakening occurs early at the articular surface, and occurs prior to the alteration of these matrix components. The extent and localization of changes in particular tissue properties set the stage for further studies with specific and graded manipulations to test the mechanistic relationship for the sequence of events described in Figure 2.4. Diminished tissue integrity, associated with aging in the LFC and with the MFC in general, appears to be an early and sensitive marker of cartilage degeneration and may causally contribute to degeneration and the development of OA.

## 2.6 Acknowledgments

This chapter was submitted to *Arthritis and Rheumatism* in October of 2004 and is currently in revision (©John Wiley & Sons, Inc.). It was reprinted with the permission of the publisher, Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. This work was supported by the Arthritis Foundation, the National Institutes of Health, and the National Science Foundation. The dissertation author (primary investigator) thanks the coauthors of the manuscript for their contributions: Won Bae, Michael Chen, David Amiel, and Richard Coutts. We thank Karen Bowden for preparation of the histological slides and Drs. Kenji Yamada, Kenji Kobayashi, Tsuyoshi Watanabe, and Hirohito Mitsuyama, UCSD, La Jolla, CA for preparing tissue samples for this study.

## 2.7 References


1. Aigner T, Hemmel M, Neureiter D, Gebhard PM, Zeiler G, Kirchner T, McKenna L: Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritic human articular knee cartilage. *Arthritis Rhuem* 44:1304-12, 2001.

2. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH: Tensile properties of human knee joint cartilage: I. influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus. *J Orthop Res* 4:379-92, 1986.

3. Bae WC, Temple MM, Amiel D, Coutts RD, Niederauer GG, Sah RL: Indentation testing of human cartilage: sensitivity to articular surface degeneration. *Arthritis Rheum* 48:3382-94, 2003.

4. Bank RA, Bayliss MT, Lafeber FPJG, Maroudas A, TeKoppele JM: Ageing and zonal variation in post-translational modification of collagen in normal human articular cartilage. The age-related increase in non-enzymatic glycation affects biomechanical properties of cartilage. *Biochem J* 330:345-51, 1998.

5. Bank RA, Krikken M, Beekman B, Stoop R, Maroudas A, Lafeber FPJG, Te Koppele JM: A simplified measurement of degraded collagen in tissues: application in healthy, fibrillated and osteoarthritic cartilage. *Matrix Biol* 16:233-43, 1997.

6. Bayliss MT, Hutton S, Hayward J, Maciewicz RA: Distribution of aggrecanase (ADAMts 4/5) cleavage products in normal and osteoarthritic human articular cartilage: the influence of age, topography and zone of tissue. *Osteoarthritis Cartilage* 9:553-60, 2001.

7. Bennett GA, Waine H, Bauer W. Changes in the knee joint at various ages with particular reference to the nature and development of degenerative joint disease. New York: The Commonwealth Fund; 1942.

8. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chan J, Van Wart H, Poole AR: Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* 99:1534-45, 1997.

9. Buckland-Wright JC, Macfarlane DG, Lynch JA, Jasani MK, Bradshaw CR: Joint space width measures cartilage thickness in osteoarthritis of the knee: high resolution plain film and double contrast macroradiographic investigation. *Ann Rheum Dis* 54:263-8, 1995.

10. Chen AC, Temple MM, Ng DM, DeGroot J, Verzijl N, TeKoppele JM, Sah RL: Induction of advanced glycation endproducts alters tensile properties of articular cartilage. *Arthritis Rheum* 46:3212-7, 2002.

11. Chen SS, Falcovitz YH, Schneiderman R, Maroudas A, Sah RL: Depth-dependent compressive properties of normal aged human femoral head articular cartilage. *Osteoarthritis Cartilage* 9:561-9, 2001.

12. Clark JM, Simonian PT: Scanning electron microscopy of "fibrillated" and "malacic" human articular cartilage: technical considerations. *Microsc Res Tech* 37:299-313, 1997.

13. Dodge GR, Poole AR: Immunohistochemical detection and immunochemical analysis of type II collagen degradation in human normal rheumatoid, and osteoarthritis articular cartilage and in explants of bovine articular cartilage cultured with interleukin 1. *J Clin Invest* 83:647-61, 1989.

14. Dudhia J, Davidson CM, Wells TM, Vynios DH, Hardingham TE, Bayliss MT: Age-related changes in the content of the C-terminal region of aggrecan in human articular cartilage. *Biochem J* 313:933-40, 1996.

15. Farndale RW, Buttle DJ, Barrett AJ: Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 883:173-7, 1986.

16. Frank EH, Grodzinsky AJ, Koob TJ, Eyre DR: Streaming potentials: a sensitive index of enzymatic degradation in articular cartilage. *J Orthop Res* 5:497-508, 1987.

17. Fujimoto D, Moriguchi T: Pyridinoline, a non-reducible crosslink of collagen. Quantitative determination, distribution, and isolation of a crosslinked peptide. *J Biochem (Tokyo)* 83:863-7, 1978.

18. Harrington IJ: A bioengineering analysis of force actions at the knee in normal and pathological gait. *Biomed Eng* 11:167-72, 1976.

19. Hayami T, Pickarski M, Wesolowski GA, McLane J, Bone A, Destefano J, Rodan GA, Duong le T: The role of subchondral bone remodeling in osteoarthritis: reduction of cartilage degeneration and prevention of osteophyte formation by alendronate in the rat anterior cruciate ligament transection model. *Arthritis Rheum* 50:1193-206, 2004.

20. Hjertquist SO, Lemperg R: Identification and concentration of the glycosaminoglycans of human articular cartilage in relation to age and osteoarthritis. *Calcif Tissue Res* 10:223-37, 1972.

21. Hjertquist SO, Wasteson A: The molecular weight of chondroitin sulfate from human articular cartilage: effects of age and osteoarthritis. *Calc Tiss Res* 10:31-7, 1972.

22. Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, Poole AR: Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. *J Clin Invest* 93:1722-32, 1994.

23. Holmes MWA, Bayliss MT, Muir H: Hyaluronic acid in human articular cartilage: age-related changes in content and size. *Biochem J* 250:435-41, 1988.

24. Jackson DS, Cleary EG: The determination of collagen and elastin. *Methods Biochem Anal* 15:25-76, 1967.

25. Kempson GE: Relationship between the tensile properties of articular cartilage from the human knee and age. *Ann Rheum Dis* 41:508-11, 1982.

26. Kempson GE, Muir H, Pollard C, Tuke M: The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. *Biochim Biophys Acta* 297:456-72, 1973.

27. Kim YJ, Sah RLY, Doong JYH, Grodzinsky AJ: Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal Biochem* 174:168-76, 1988.

28. Lyyra T, Kiviranta I, Vaatainen U, Helminen HJ, Jurvelin JS: In vivo characterization of indentation stiffness of articular cartilage in the normal human knee. *J Biomed Mater Res* 48:482-7, 1999.

29. Maroudas A, Bayliss MT, Venn MF: Further studies on the composition of human femoral head cartilage. *Ann Rheum Dis* 39:514-23, 1980.

30. McGowan KB, Kurtis MS, Lottman LM, Watson D, Sah RL: Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen® and Hoechst 33258. *Osteoarthritis Cartilage* 10:580-7, 2002.

31. Meachim G: Light microscopy of Indian ink preparations of fibrillated cartilage. *Ann Rheum Dis* 31:457-64, 1972.

32. Meachim G, Denham D, Emery IH, Wilkinson PH: Collagen alignments and artificial splits at the surface of human articular cartilage. *J Anat* 118:101-18, 1974.

33. Mitrovic D, Quintero M, Stankovic A, Ryckewaert A: Cell density of adult human femoral condylar articular cartilage. *Lab Invest* 49:309-16, 1983.

34. Morrison JB: The mechanics of the knee joint in relation to normal walking. *J Biomech* 3:51-61, 1970.

35. Muehleman C, Bareither D, Huch K, Cole AA, Kuettner KE: Prevalence of degenerative morphological changes in the joints of the lower extremity. *Osteoarthritis Cartilage* 5:23-37, 1997.

36. Oegema TR, Carpenter BJ, Thompson RC: Fluorometric determination of DNA in cartilage of various species. *J Orthop Res* 1:345-51, 1984.

37. Plaas AHK, West LA, Wong-Palms S, Nelson FR: Glycosaminoglycan sulfation in osteoarthritis: alterations at the non-reducing termini of chondroitin and dermatan sulfate. *J Biol Chem* 273:12642-9, 1998.

38. Plaas AHK, Wong-Palms S, Roughley PJ, Midura RJ, Hascall VC: Chemical and immunological assay of the nonreducing terminal residues of chondroitin sulfate from human aggrecan. *J Biol Chem* 272:20603-10, 1997.

39. Poole AR: Imbalances of anabolism and catabolism of cartilage matrix components in osteoarthritis. In: *Osteoarthritic Disorders,* ed. by KE Kuettner, Goldberg VM, American Academy of Orthopaedic Surgeons, Rosemont, IL, 1995, 247-60.

40. Poole AR, Kobayashi M, Yasuda T, Laverty S, Mwale F, Kojima T, Sakai T, Wahl C, El-Maadawy S, Webb G, Tchetina E, Wu W: Type II collagen degradation and its regulation in articular cartilage in osteoarthritis. *Ann Rheum Dis* 61S2:78-81, 2002.

41. Praemer A, Furner S, Rice DP. Musculoskeletal conditions in the United States. Park Ridge, IL: American Academy of Orthopaedic Surgeons; 1999.

42. Pullig O, Weseloh G, Ronneberger D, Kakonen S, Swoboda B: Chondrocyte differentiation in human osteoarthritis: expression of osteocalcin in normal and osteoarthritic cartilage and bone. *Calcif Tissue Int* 67:230-40, 2000.

43. Quintero M, Mitrovic DR, Stankovic A, de Seze S, Miravet L, Ryckewaert A: Aspects cellulaires du vieillissement du cartilage articulaire. I. cartilage condylien a surface normale, preleve dans les genoux normaux. *Revue du Rhumatisme* 51:375-9, 1984.

44. Quintero M, Mitrovic DR, Stankovic A, de Seze S, Miravet L, Ryckewaert A: Aspects cellulaires du vieillissement du cartilage articulaire. II. cartilage condylien a surface fissuree preleve dans les genoux normaux et arthrosiques. *Revue du Rhumatisme* 51:445-9, 1984.

45. Sandy JD, Neame PJ, Boynton RE, Flannery CR: Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. *J Biol Chem* 266:8683-5, 1991.

46. Sell DR, Monnier VM: Structure elucidation of a senescence cross-link from human extracellular matrix. *J Biol Chem* 264:21597-602, 1989.

47. Shapiro F, Glimcher MJ: Induction of osteoarthrosis in the rabbit knee joint. Histologic changes following menisectomy and meniscal lesions. *Clin Orthop* 147:287-95, 1980.

48. Sokal RR, Rohlf FJ. Biometry. 3rd ed. New York: WH Freeman and Co.; 1995.

49. Stockwell RA: The cell density of human articular and costal cartilage. *J Anat* 101:753-63, 1967.

50. Treppo S, Koepp H, Quan EC, Cole AA, Kuettner KE, Grodzinsky AJ: Comparison of biomechanical and biochemical properties of cartilage from human knee and ankle pairs. *J Orthop Res* 18:739-48, 2000.

51. Uchiyama A, Ohishi T, Takahashi M, Kushida K, Inoue T, Fujie M, Horiuchi K: Fluorophores from aging human articular cartilage. *J Biochem Tokyo* 110:714-8, 1991.

52. Urban MR, Fermor B, Lee RB, Urban JP: Measurement of DNA in intervertebral disc and other autofluorescent cartilages using the dye hoechst 33258. *Anal Biochem* 262:85-8, 1998.

53. Walker GD, Fischer M, Gannon J, Thompson RC, Oegema TR: Expression of type-X collagen in osteoarthritis. *J Orthop Res* 13:4-12, 1995.

54. Williamson AK, Chen AC, Masuda K, Thonar EJ-MA, Sah RL: Tensile mechanical properties of bovine articular cartilage: variations with growth and relationships to collagen network components. *J Orthop Res* 21:872-80, 2003.

55. Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 93:440-7, 1961.

56. Woessner JF: Imbalance of proteinases and their inhibitors in osteoarthritis. In: *Osteoarthritic Disorders,* ed. by KE Kuettner, Goldberg VM, American Academy of Orthopaedic Surgeons, Rosemont, IL, 1995, 281-90.

# CHAPTER 3

# BIOMECHANICAL WEAKENING OF HUMAN ARTICULAR CARTILAGE OF THE FEMORAL CONDYLE IN EARLY DEGENERATION

## 3.1 Abstract

Objective. To characterize, in age-matched adult macroscopically normal and mildly degenerate human articular cartilage taken from different depths in the lateral and medial condyles the (1) tensile biomechanical properties, (2) density of cells, (3) content of extracellular matrix components, and (4) structure of the articular surface.

Methods. Age-matched articular cartilage samples were obtained that were macroscopically normal (NL) or mildly degenerate and from donors who were (OA) or were not (DGN) diagnosed with knee osteoarthritis. The cartilage was analyzed for changes in tensile integrity, surface wear, cell density, matrix composition, degradation, and intrinsic fluorescence.

Results. DGN and OA cartilage had lower tensile integrity than NL cartilage which coincided with a high amount of surface wear, lower fluorescence, and a higher amount of collagen degradation. High water content was a feature of OA samples which may have contributed to low measures of cell density and collagen and glycosaminoglycan content.

Conclusions. Changes in these properties suggested that dramatic changes were occurring in the collagen network contributing to the loss of tensile integrity. Many of the changes seen here in mildly degenerate articular cartilage are identical to those regarded as features of osteoarthritis. These results provide support for the roles of mechanical wear, enzymatic degradation, and collagen network remodeling as mediators in the progression of early cartilage degeneration and osteoarthritis.

## 3.2 Introduction

The tensile integrity of human articular cartilage diminishes markedly with the onset of early cartilage degeneration. In particular, in the superficial layer, the equilibrium tensile modulus of articular cartilage of the medial femoral condyle has been shown to be ~70 and 85% lower in cartilage adjacent to mild fibrillation and osteoarthritic lesions, respectively [3] when compared to macroscopically normal articular cartilage. Samples in that study were, however, not age-matched, with normal samples from young donors (33±4 yrs old) and mildly fibrillated and osteoarthritic specimen from middle (56±4 yrs old) and old (69±3 yrs old) age donors, respectively. Thus, it was unclear what role age-associated tensile weakening had in the diminution of the tensile modulus in that study. In addition, it is, as yet, unknown what changes in the surface structure and biochemical constituents of articular cartilage may contribute to the loss of tensile integrity with early degeneration.

Mildly degenerate cartilage exhibits many differences from macorscopically normal cartilage in the cell density, extracellular matrix constituents, and surface structure which may contribute to biomechanical weakening. The cell density of adult articular cartilage is lower in fibrillated cartilage [41, 47]. This may coincide with an increase in empty lacunae in osteoarthritic cartilage, but it is unclear if it is a result of cell death or cell loss due to histological preparation as a previous study [1] found a negligible number of empty lacunae in osteoarthritic cartilage and <1% of osteoarthritic cells undergoing apoptotic cell death. A decrease in cell density could contribute to the inability of remaining cells to maintain matrix homeostasis.

Enzymatic degradation of collagen and proteoglycans could also contribute to biomechanical weakening of mildly fibrillated and osteoarthritic cartilage. Increased water content of articular cartilage is a hallmark of early osteoarthritis [33, 37] and results from proteoglycan molecules swelling within a degraded collagen network [9, 33, 37]. The production of pro-inflammatory cytokines and proteases in osteoarthritis [52, 56, 59] may cause the fragmentation and loss of collagen molecules [11, 16] and proteoglycans [17, 49]. Aggrecan fragments accumulate with age in normal articular cartilage [55], and, in cartilage displaying signs of early cartilage degeneration are even smaller [48]. Increased cleavage and denaturation of collagen molecules has been demonstrated in osteoarthritic cartilage [11, 16, 24, 25]. Enzymatic collagen degradation has also been shown to dramatically reduce the dynamic tensile stiffness and strength of articular cartilage [5, 30, 31]. Thus, the degradation of extracellular matrix molecules may contribute directly to mechanical weakening.

Collagen network remodeling, indicated by a loss of intrinsic cartilage matrix fluorescence, may also contribute to biomechanical weakening of mildly degenerate cartilage. Background fluorescence results from the presence of products of collagen glycation or advanced glycation end products (AGE), such as pentosidine. Their presence in articular cartilage is known to increase with age after skeletal maturity [7], and is associated with a lack of collagen turnover in cartilage [36]. In osteoarthritic cartilage, a loss of this intrinsic fluorescence has been observed and suggested to be associated with collagen degradation [21] as well as an induction of a chondrocyte phenotype associated with immature cartilage [20]. It may be a combination of

collagen degradation and loss of intrinsic cartilage fluorescence which contributes to the tensile biomechanical weakening observed in mildly degenerate articular cartilage.

Cartilage weakening could directly reflect mechanical wear of the articular surface. Wear of the articular surface has been evidenced as increased India ink uptake on the articular surface [39]. The area of ink-staining has been shown to increase with age [40] but may have been a combination of mild age-associated surface alterations and severe osteoarthritic cartilage erosion. The associated cartilage weakening and mechanical wear may be a manifestation of physical disruption of the collagen network, especially at or near the articular surface [14]. It could result in a mechanically weak articular surface with possible consequences of further cell death or altered cell biosynthesis and detrimental effects on the quality of extracellular matrix.

Identification of changes that occur structurally or biochemically in the progression from macroscopically normal cartilage to cartilage displaying signs of early degeneration may give an idea of when and why such changes progress to OA disease. It was the hypothesis of this study that human articular cartilage showing signs of early degeneration, exhibits tensile weakening in a depth- and site-associated manner that is due to a composition and structure that is distinctly different from that of macroscopically normal articular cartilage. The objectives were to characterize, in age-matched adult macroscopically normal and mildly degenerate human articular cartilage taken from different depths at the LFC and MFC sites, (1) tensile biomechanical properties, (2) density of cells, (3) content of extracellular matrix components, and (4) structure of the articular surface.

## 3.3 Materials and Methods

### 3.3.1. Sample Selection and Preparation

Age-matched samples (41-91 yrs, 63±1 yrs (mean±SEM), Table 3.1) in the form of 10-mm osteochondral cores were selected from the anterior region of the MFC ($n$=35) and LFC ($n$=32). Some samples were from forty-five cadaveric human donors obtained from tissue banks with donation areas in the Western and Southern areas of the United States. These were grouped according to a macroscopic surface appearance at the time of harvest that was either smooth (normal, NL, n=40) or roughened (degenerate, DGN, n=12). Additional samples were obtained from twelve patients undergoing total knee arthroplasty. These samples had a roughened surface appearance and were adjacent to regions of cartilage erosion resulting in exposure of subchondral bone (OA, n=15). As a measure of the severity of osteoarthritic disease, the area of full thickness cartilage erosion of the femoral compartment was measured and found to be significantly higher in OA samples than NL or DGN samples (each $p<0.005$, Table 3.1). In this grouping configuration, the mean age of donors of LFC and MFC samples from each experimental group was similar ($p=0.08$-$0.2$), but the body mass index (BMI) was slightly higher in OA than NL donors of LFC and MFC samples (each $p<0.05$, Table 3.1).

### 3.3.2. Structural Indices of Wear

Portions of these samples were examined for cartilage thickness, histopathology (Mankin-Shapiro score, surface irregularity), and surface roughening (reflectance score after India ink staining) exactly as described previously [6].

| | | Lateral Femoral Condyle | | | Medial Femoral Condyle | | |
|---|---|---|---|---|---|---|---|
| | | NL | DGN | OA | NL | DGN | OA |
| Full thickness erosion of femoral compartment ($mm^2$) | | 0 | 80±40 | 280±70$^{**,++}$ | 0 | 70±40 | 331±70$^{**,++}$ |
| Age (yr) | Female | 63±6 | 72±8 | 71±3 | 60±5 | 67±9 | 69±3 |
| | Male | 58±3 | 59±2 | 52 | 58±3 | 70±8 | 65±6 |
| | Female+Male | 60±3 | 68±6 | 69±5 | 59±3 | 69±5 | 68±3 |
| BMI ($kg/m^2$) | Female | 22±2 | 22±4 | 30±1 | 19±1 | 23±5 | 30±1 |
| | Male | 24±1 | 38±10 | 32 | 24±1 | 28±1 | 25±1 |
| | Female+Male | 23±1 | 27±5 | 30±1$^{*}$ | 22±1 | 25±3 | 28±1$^{*}$ |
| *n* | Female | 8 | 4 | 7 | 7 | 3 | 5 |
| | Male | 13 | 2 | 1 | 12 | 3 | 2 |
| | Female+Male | 21 | 6 | 8 | 19 | 6 | 7 |

**Table 3.1:** Donor description. Area of full thickness cartilage erosion of femoral compartment, age, body mass index (BMI), and *n* of human female and male donors of osteochondral cores from the LFC and MFC reported as mean ± SEM. *$p<0.05$, **$p<0.005$ versus NL, $^{++}p<0.005$ versus DGN.

### 3.3.3. Biomechanical Properties

Portions of each core were separated into superficial, middle, and deep layers and analyzed by tensile testing. Briefly, the cartilage of each core was sliced into ~0.3-mm thick layers, at a distance from the articular surface of 0% (superficial layer, including the articular surface), 30% (middle layer), and 60% (deep layer) of the average cartilage thickness. The slices were cut into tapered specimens for equilibrium and constant strain-rate tensile testing [57]. The equilibrium test results were analyzed to determine tensile equilibrium modulus, and the dynamic test results were used to assess tensile ramp modulus, tensile strength, and failure strain.

### 3.3.4. Biochemical Properties

Portions of tissue slices, adjacent to tensile samples, were analyzed for cell and matrix properties. Briefly, these portions were weighed wet, lyophilized, weighed dry, and solubilized with proteinase K (ProK). These solubilized portions were used to determine total DNA content [38], hydroxyproline content [58], degraded collagen content (COL in aCT, [8]), intrinsic fluorescence [38], and sulfated glycosaminoglycan (GAG) content [19]. DNA was converted to cell number using a conversion factor of 7.3 pg DNA/human chondrocyte [51]. Hydroxyproline content was converted to collagen (COL) content using a mass ratio of collagen to hydroxyproline equal to 7.1 [27]. Intrinsic fluorescence was measured at excitation (Ex) and emission (Em) wavelengths corresponding to maximum fluorescence of pyridinoline (Ex 295/Em 395 nm) and pentosidine (Ex 335/Em 385 nm) crosslinks. The intrinsic fluorescence was reported as a ratio of pentosidine-associated fluorescence to pyridinoline-associated fluorescence (fluorescence ratio). Sulfated

GAG content was calculated by comparison to known concentrations of shark chondroitin sulfate. The contents of DNA, COL, and GAG were calculated as the mass normalized to wet weight.

### 3.3.5. Statistics

The effect of degeneration on the various mechanical and biochemical parameters was assessed using repeated measures ANOVA with anatomical location (LFC or MFC) and depth from the surface (superficial, middle, or deep) as repeated factors. When anatomical location was found to have a significant effect ($p<0.05$), the locations were analyzed separately. When experimental group or depth from the articular surface had an effect (for $p<0.05$), planned comparisons were made between experimental groups at each depth or between depths for each experimental group. To limit the experimentwise error rate, each comparison was tested using a significance level alpha = 0.05 divided by the number of comparisons made [50]. For results that were expressed as a percentage, data were arcsine transformed to improve normality, prior to the above statistical analyses. For ordinal data (i.e., histopathology index and surface irregularity), the effect of location was tested using the Mann-Whitney U test, and the effect of experimental group within each of the LFC and MFC locations was analyzed with the Kruskal-Wallis test and Dunn's test. All data are reported as mean ± SEM.

## 3.4 Results

**Structural Indices of Wear.** Samples selected for this study had surface structural properties that were dependent on anatomical location (site) and depth (layer) and reflected cartilage that had either a smooth, macroscopically normal surface or a mildly fibrillated cartilage surface. The overall cartilage thickness (Figure 3.1a) was similar between NL, DGN, and OA groups ($p=0.6$), as well as between LFC and MFC sites ($p=0.4$). Mild cartilage surface fibrillation was evidenced by increased ink-staining (lower reflectance score) in DGN and OA samples compared to NL samples (Figure 3.1b). The reflectance score was ~10% lower at MFC than LFC sites ($p<0.05$). In the LFC, the reflectance score was higher in NL samples compared to DGN and OA samples (each, $p<0.005$), while in the MFC, NL, DGN, and OA samples had similar, low reflectance scores ($p=0.2$-$0.8$). The variance of reflectance score was similar between the LFC and MFC sites ($p=0.6$) but was dependent on experimental group (Figure 3.1c). In the LFC, the variance tended to be higher in both DGN ($p=0.05$) and OA ($p<0.005$) samples than that of NL samples. However, in the MFC, the variance was similarly high between NL and DGN ($p=0.6$), as well as OA samples ($p=1.0$). Consistent with the sample selection of macroscopically normal (NL) and mildly fibrillated cartilage samples (DGN, OA), the histopathology score (Figure 3.1d), while not dependent on site ($p=0.1$), was higher in DGN and OA samples than NL samples at LFC ($p<0.005$, $p<0.01$, respectively) and MFC ($p<0.005$, $p<0.05$, respectively) sites. The increase in the semi-quantitative histopathological assessment of surface irregularity (Figure 3.1e) paralleled the pattern of decrease in reflectance score and increase in histopathology index, being ~28% higher at MFC than LFC sites

and being markedly higher in the LFC in DGN and OA samples than NL samples (each, $p<0.005$). Taken together, these data show that while NL samples were judged to be macroscopically normal with a smooth articular surface, very mild site-associated surface fibrillation was evident, and in DGN and OA samples, samples judged to have macroscopic surface fibrillation, the surface degeneration was more severe than in NL samples but did not affect the overall cartilage thickness.

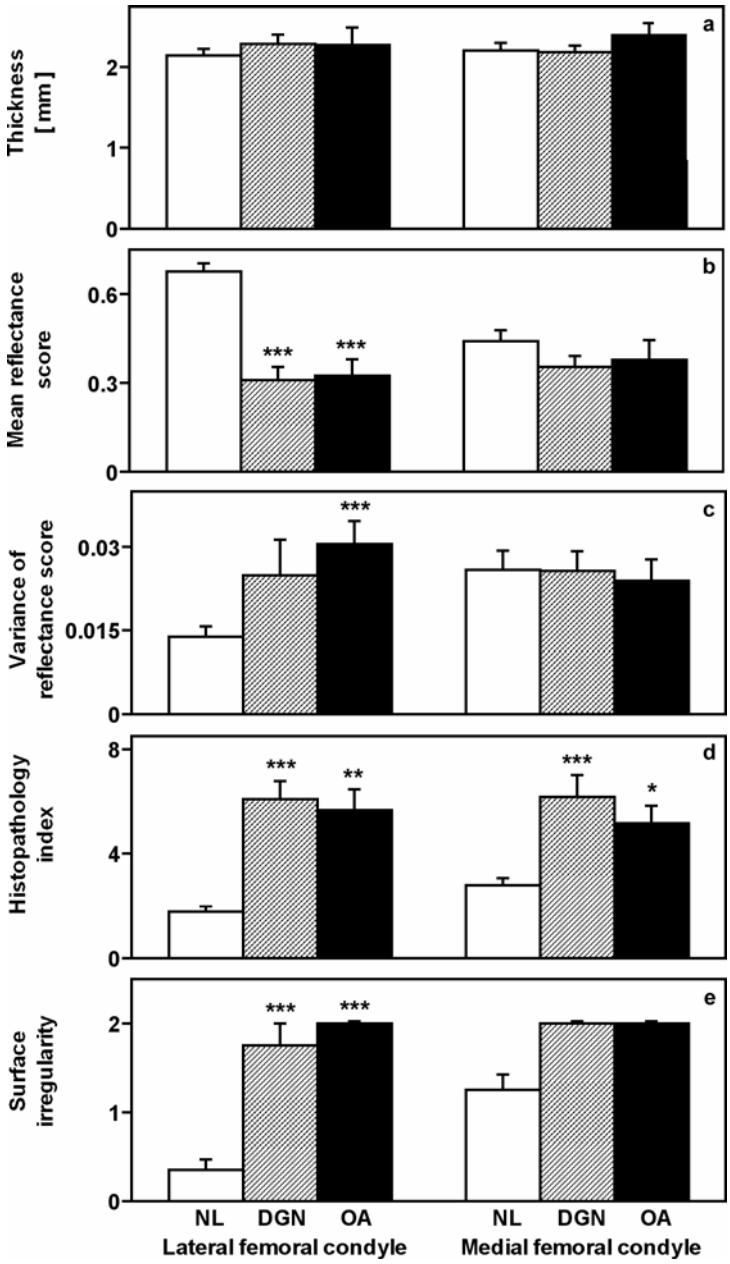



**Figure 3.1:** Structural and surface properties of human articular cartilage from the LFC and MFC. Cartilage thickness (**a**), reflectance score assessed after India ink staining (**b**), variance of reflectance score (**c**), overall histopathological index of cartilage degeneration (**d**) and surface irregularity assessed by histopathological grading (**e**) from age-matched donors of cartilage with articular surfaces that were macroscopically normal (NL, *n*=19-21), mildly fibrillated (DGN, *n*=6), and mildly fibrillated from joints undergoing total knee replacement (OA, *n*=7-8). *p<0.05, **p<0.01, ***p<0.005 versus NL samples.

**Biomechanical Properties.** The variation in mechanical properties of these age-matched NL, DGN, and OA samples reflects a dramatic shift in the equilibrium and dynamic response of cartilage to load, independent of any age-associated decrease in tensile properties. The variation in mechanical properties was dependent on depth and anatomical location. The tensile ramp modulus (Figure 3.2b), strength (Figure 3.2c) and failure strain (Figure 3.2d) were lower in the MFC than the LFC (each, $p<0.05$), while the equilibrium modulus (Figure 3.2a) was similar between sites ($p=0.3$). Ramp modulus, strength, and failure strain were, and equilibrium modulus tended to be, depth-dependent ($p<0.005$, $p<0.01$, $p<0.005$, and $p=0.1$, respectively), with the ramp modulus being 124% higher, the strength being 10% higher, the failure strain being 52% lower, and the equilibrium modulus being 80% higher in the superficial than the deep layer. Due to the low tensile integrity of DGN and OA cartilage, the depth-dependence of the tensile properties was not as evident in those groups. Despite this, specific group differences were outlined below as a function of site and depth.

In the LFC, the pattern of loss of tensile integrity with experimental group was such that the superficial layer was most affected, followed by the middle and deep layers. In the superficial layer, the tensile integrity was higher in the NL than either the DGN or OA groups. Tensile equilibrium modulus, ramp modulus, strength, and failure strain were or tended to be higher in NL than DGN ($p<0.005$, $p<0.005$, $p<0.005$, $p<0.01$, respectively) and OA groups ($p<0.005$, $p=0.07$, $p<0.005$, $p=0.04$, respectively). In the middle layer, the strength and failure strain were or tended to be

higher in NL than DGN (each $p<0.005$) and OA samples ($p=0.05$, $p<0.005$, respectively), while equilibrium and ramp moduli were similar between groups ($p=0.1$-$1.0$). In the deep layer, the equilibrium modulus ($p=0.5$-$1.0$), ramp modulus ($p=0.4$-$0.8$) and strength ($p=0.05$-$0.5$) were similar between experimental groups, while the failure strain was higher in NL than either DGN ($p<0.005$) or OA ($p<0.005$) groups.

In the MFC, the tensile integrity was low in all groups, relative to the LFC. In the superficial layer, the equilibrium modulus, strength, and failure strain, but not ramp modulus were higher in NL than either DGN ($p<0.01$, $p<0.01$, $p<0.025$, $p=0.2$, respectively) or OA samples ($p=0.04$, $p<0.025$, $p<0.025$, $p=0.6$, respectively). In the middle layer, the equilibrium and ramp moduli were similar between groups ($p=0.1$-$0.4$), while the strength and failure strain tended to be higher in NL than DGN ($p=0.05$, $p=0.07$, respectively) or OA samples ($p=0.2$, $p<0.025$, respectively). Similarly, in the deep layer, the equilibrium and ramp moduli were similar between groups ($p=0.2$-$0.9$), while the strength and failure strain tended to be higher in NL than DGN ($p<0.01$, $p<0.005$, respectively) or OA samples ($p<0.01$, $p<0.005$, respectively). In all, the variation in tensile integrity between NL, DGN, and OA cartilage samples reflects distinct patterns of tensile weakening, independent of and beyond age-associated cartilage weakening.

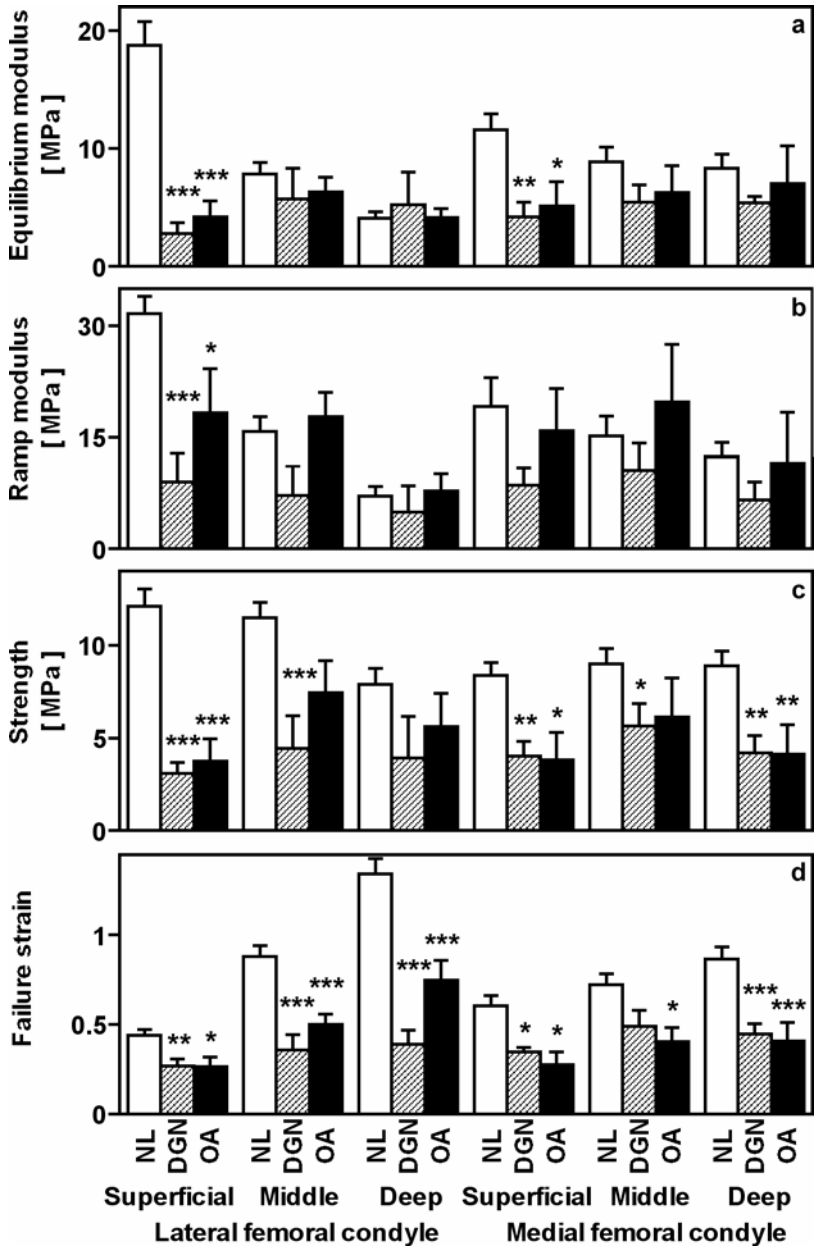



**Figure 3.2:** Tensile biomechanical properties of samples described in Figure 3.1. For specimens from the superficial, middle, and deep layers, the tensile equilibrium modulus (**a**), tensile ramp modulus (**b**), tensile strength (**c**), and failure strain (**d**) were determined from equilibrium and then non-equilibrium failure testing of articular cartilage from age-matched NL, DGN, and OA donors. *p<0.05, **p<0.01, ***p<0.005 versus NL samples.

**Biochemical Properties.** Variations in biochemical constituents with experimental group were dependent on depth and tended not to depend on site. The contents of water (Figure 3.3a), DNA (Figure 3.3b), COL (Figure 3.3c), COL in aCT (Figure 3.3d), and GAG (Figure 3.3f) were not dependent on site ($p=0.06$, $p=0.4$, $p=0.05$, $p=0.4$, and $p=1.0$, respectively). The fluorescence ratio (Figure 3.3e), however, was 11% higher in the LFC than the MFC ($p<0.05$). The water ($p<0.01$), DNA ($p<0.005$), COL in aCT ($p<0.005$), and GAG ($p<0.005$) contents were dependent on depth while the COL content ($p=0.9$) and the fluorescence ratio ($p=0.1$) were not. In particular, water, DNA, and COL in aCT were higher and GAG was lower by 5%, 140%, 17%, and 63%, respectively, in the superficial than the deep layer. Distinct site- and depth-dependent variations between experimental groups are detailed below.

The water content was higher in OA cartilage than NL and DGN cartilage in all layers of both the LFC and MFC. In the LFC, water content (Figure 3.3a) was higher in OA than NL samples and tended to be higher than DGN samples in superficial ($p<0.005$, $p=0.1$, respectively), middle ($p=0.05$, $p=0.7$, respectively), and deep layers ($p=0.03$, $p=0.2$, respectively). Similarly, in the MFC, the water content (Figure 3.3a) was higher in OA than NL samples and tended to be higher than DGN samples in superficial ($p<0.01$, $p=0.1$, respectively), middle ($p<0.025$, $p=0.08$, respectively), and deep layers ($p<0.005$, $p<0.025$, respectively). Increased water content, a hallmark of osteoarthritis, was evident in samples with early OA.

Variations in DNA content indicated that cellular changes were depth dependent, being most dramatic in the superficial layer. In the LFC, DNA content

(Figure 3.3b) was lower in the superficial layer of DGN and OA samples (each $p<0.005$) compared to that of NL samples, but was not different in the middle or deep layers ($p=0.1$-$0.6$). In the MFC, DNA content (Figure 3.3b) in the superficial and middle layers was lower in OA samples than in NL samples ($p<0.005$, $p=0.03$, respectively) and tended to be lower in OA than DGN samples ($p=0.04$, $p=0.1$, respectively), while in the deep layer, there was little variation between experimental groups ($p=0.6$-$0.9$).

Changes in the collagen network were evidenced as alterations in COL, COL in aCT, and intrinsic fluorescence (fluorescence ratio). In the LFC, there was a tendency for the COL content (Figure 3.3c) of NL samples to be higher than DGN and OA samples in superficial ($p=0.05$, $p=0.06$, respectively), middle ($p=0.1$, $p<0.01$, respectively), and deep layers ($p=0.08$, $p<0.025$, respectively) layers. In the MFC, COL content (Figure 3.3c) was mildly lower in the superficial layer of OA samples than NL samples ($p<0.025$), but varied little in the middle and deep layers ($p=0.1$-$1.0$). COL in aCT (Figure 3.3d) varied with site, with DGN and OA samples of the LFC tending to be higher than NL samples in superficial ($p<0.005$, $p=0.03$, respectively), middle ($p<0.005$, $p=0.07$, respectively), and deep layers (each, $p=0.1$). In the MFC, the OA samples displayed a notable amount of COL in aCT (Figure 3.3d), being higher than NL and DGN samples in superficial ($p<0.005$, $p<0.01$, respectively), middle ($p<0.025$, $p=0.3$, respectively), and deep ($p<0.01$, $p=0.06$, respectively) layers. The amount of COL in aCT varied greatly from sample to sample and may have been related to the proximity to areas of full thickness cartilage erosion and size of those regions in the respective LFC or MFC compartment. There was a striking decrease in

the fluorescence ratio, being more pronounced in the LFC than MFC. In the LFC, the fluorescence ratio (Figure 3.3e) was lower in DGN and OA samples than in NL samples in superficial, middle, and deep layers (each, $p<0.005$). In the MFC, the fluorescence ratio (Figure 3.3e) was lower in DGN and OA samples than in NL samples in the superficial layer (each, $p<0.005$) and tended to be lower in the middle ($p=0.04$, $p=0.06$, respectively) and deep (each, $p=0.025$) layers. The changes in the structure of the collagen network, especially the fluorescence ratio, which is an indicator of collagen crosslinking, may indicate a collagen network undergoing remodeling and coincides with the decrease in tensile integrity of cartilage displaying signs of early degeneration.

There was a mild decrease in GAG content with the onset of early cartilage degeneration. In the LFC, the GAG content (Figure 3.3f) in the superficial and middle layers was mildly higher in NL samples than DGN ($p=0.2$, $p=0.4$, respectively) and OA samples (each $p=0.1$), but not in the deep layer ($p=0.8$-$1.0$). In the MFC, the GAG content (Figure 3.3f) tended to be higher in the superficial layer of NL samples than DGN ($p=0.04$) and OA samples ($p=0.1$). In the middle and deep layers, the GAG content of NL samples tended to be higher than OA (each $p=0.2$), but not DGN samples ($p=0.6$-$0.9$). Alterations in certain biochemical constituents were consistent with changes observed in early OA, and coincided with tensile weakening observed in samples displaying signs of early cartilage degeneration.

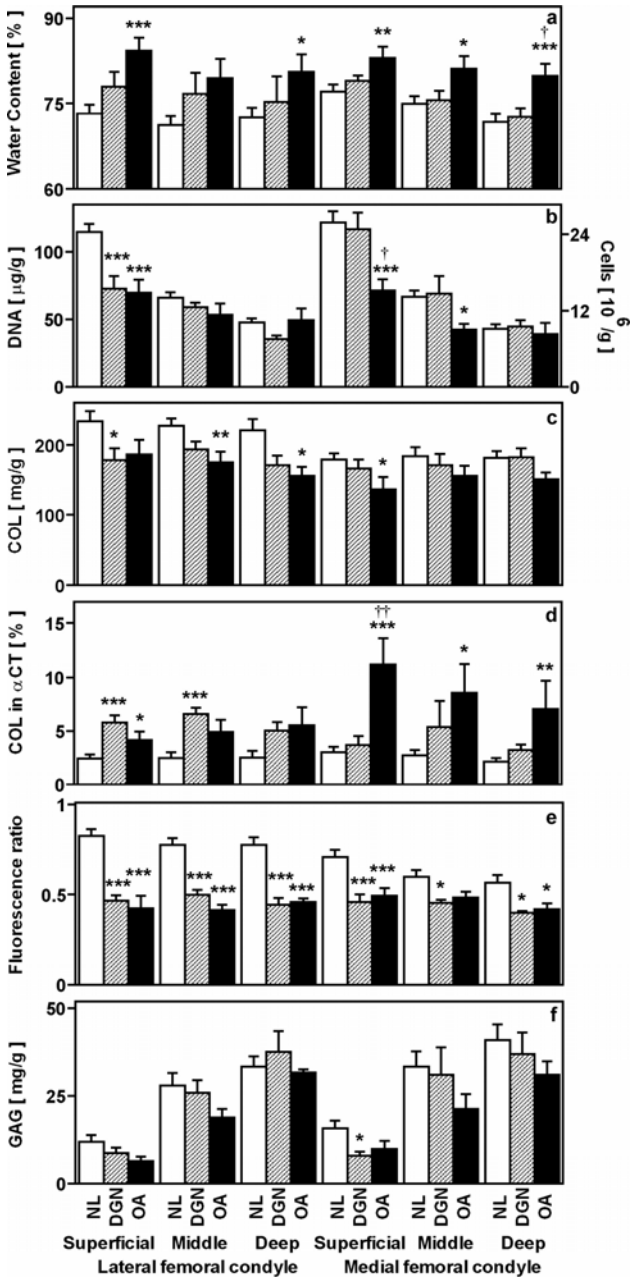


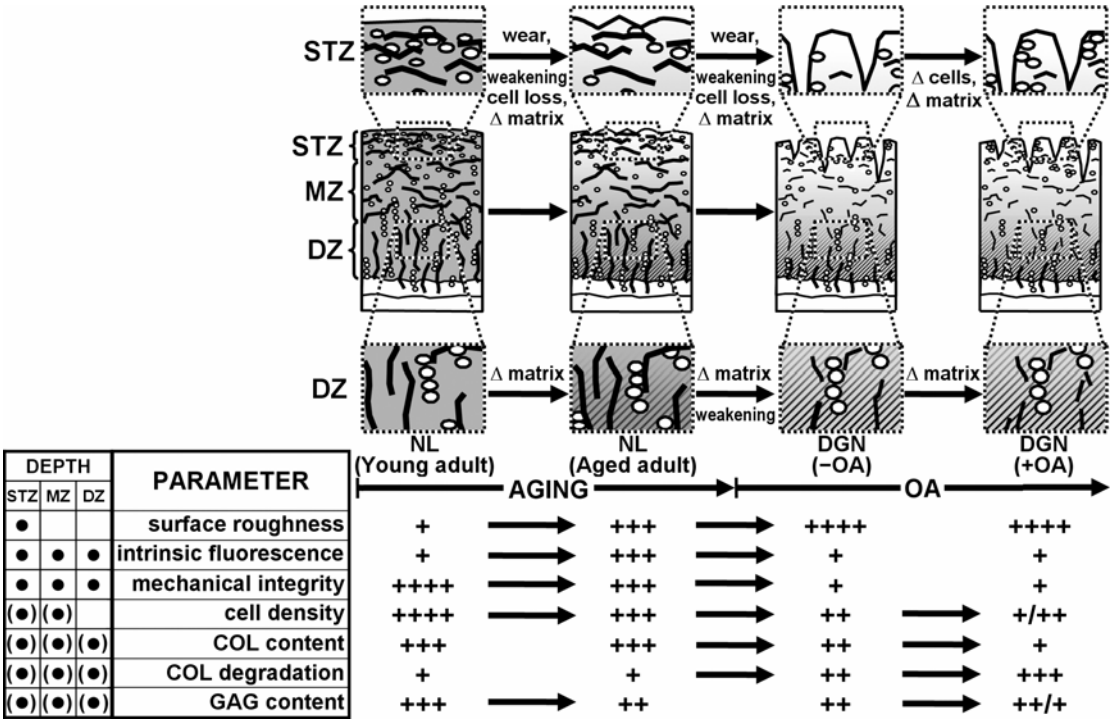

**Figure 3.3:** Biochemical properties of human articular cartilage samples described in Figure 3.1. Cartilage tissue adjacent to the mechanical test specimens was analyzed for water content (**a**), DNA and calculated cell number (**b**), COL (**c**), COL in aCT (**d**), the fluorescence ratio of pentosidine-associated fluorescence (Ex 355/Em 385 nm) to pyridinoline-associated fluorescence (Ex 295/Em 395 nm) (**e**), and GAG (**f**). DNA, COL, and GAG were each normalized to wet weight. *p<0.05, **p<0.01, ***p<0.005 versus NL samples. †p<0.05, ††p<0.01 versus DGN samples.

## 3.5 Discussion

This study detailed the severe loss of cartilage integrity that occurs with the onset of early cartilage degeneration, independent of age-associated cartilage weakening. The results highlighted a sequence of changes that occurred with the advancement of cartilage degeneration and that was prominent at certain anatomical locations and depths. Markedly lower tensile strength and failure strain in DGN and OA than NL samples were noted in superficial, middle, and deep layers (Figure 3.2c, d), as well as a lower equilibrium modulus in the superficial layer (Figure 3.2a) at both the LFC and MFC sites. There were changes in structure and biochemical constituents occurring parallel to changes in tensile integrity (Figure 3.4). An increase in surface wear was evidenced in the LFC as a lower mean reflectance score (Figure 3.1b) and a higher variance of the reflectance score in DGN and OA than NL samples (Figure 3.1c) and in both the LFC and MFC as a higher histopathology index (Figure 3.1d) with the surface irregularity being a chief contributor to that increase (Figure 3.1e). There were also changes in matrix components, notably the collagen network. Higher COL in aCT (Figure 3.3c), and a lower fluorescence ratio (Figure 3.3d) in DGN and OA than NL samples point to changes in the collagen network which could trigger the tensile weakening seen in DGN and OA samples. A higher water content (Figure 3.3a), lower cell density, and lower GAG and COL contents in OA samples followed the changes in the tensile properties, the surface structure, and the collagen network. This study provides the initial measures of surface wear and alteration of matrix components in age-matched macroscopically normal and mildly degenerate articular

cartilage which may be involved in the progression of tensile weakening in early cartilage degeneration.

Variations in structural, biomechanical, and biochemical properties observed between the LFC and MFC sites may reflect intrinsic site-associated differences in weight-bearing. At the MFC site, there was a higher amount of surface wear (low mean reflectance score, high variance of reflectance score, and high histopathology score) in NL samples than in the LFC. Corresponding to that, the NL samples were weaker in the superficial layer of the MFC than the LFC, with DGN and OA samples being similarly weak at both sites. In addition, there was a higher amount of collagen degradation in OA samples and lower intrinsic fluorescence in samples of the MFC. The medial compartment of the knee is subjected to higher joint pressures than the lateral [22, 42], a loading pattern that may cause earlier and more severe degenerative changes at the MFC than LFC sites [10, 15, 43]. These features highlight differences in the function, composition, and structure of articular cartilage that may provide insight into the mechanisms of OA disease-associated weakening.




| DEPTH | | | PARAMETER | | | | | |
|---|---|---|---|---|---|---|---|---|
| STZ | MZ | DZ | | | | | | |
| ● | | | surface roughness | + | +++ | ++++ | | ++++ |
| ● | ● | ● | intrinsic fluorescence | + | +++ | + | | + |
| ● | ● | ● | mechanical integrity | ++++ | +++ | + | | + |
| (●) | (●) | | cell density | ++++ | +++ | ++ | | +/++ |
| (●) | (●) | (●) | COL content | +++ | +++ | ++ | | + |
| (●) | (●) | (●) | COL degradation | + | + | ++ | | +++ |
| (●) | (●) | (●) | GAG content | +++ | ++ | ++ | | ++/+ |

**Figure 3.4:** Summary of cartilage changes with age and early cartilage degeneration. Changes in superficial tangential (STZ), middle zone (MZ) and deep zone (DZ) are denoted by ●, with parentheses signifying variable changes. Depicted are changes in mechanical integrity (degree of gray shading), articular surface wear, alterations of chondrocyte density, collagen network alteration (fragmented fibrils and loss of fluorescence), and loss of GAG (▨).

Interpretation of results of this study must be done with certain considerations in mind. Variations between human donors due to genetics, lifestyle, and diet could not be controlled for and may be especially important considering a donor with OA may be more sedentary (have a higher BMI) due to activity limitations associated with joint pain and disability. Similarly, the selection of mildly degenerate samples and grouping them as being from joints that were (OA) and were not (DGN) diagnosed with OA could lead to sample variability within those groups because many people with radiographic evidence of OA have no symptoms or disability and may go undiagnosed [32]. However, joints from patients with OA in this study had larger regions of full thickness cartilage erosion (Table 3.1), indicating that the severity of cartilage degeneration in those donors was higher. Attempts were made to minimize the effects of such factors by selecting samples from the same anterior LFC and MFC sites and selecting samples of a similar age range.

Measures of tensile properties in this study expanded upon those of previous studies by assessing the effect of degeneration in age- and site-matched samples. Age and site could have confounding effects on a study of cartilage degeneration because of the prevalence of OA at advanced age [46] and because cartilage degeneration occurs earlier and is more severe in the medial than the lateral femoral condyle [10, 15, 43]. Values for tensile moduli, strength, and failure strain were within the range of values obtained previously in studies of tensile equilibrium [3, 4] and dynamic failure [29, 30] for aged, macroscopically normal and mildly degenerate articular cartilage but may be more representative of the adult population as it includes a larger sample size. The ~85 and 70% lower tensile equilibrium modulus of middle-age mildly

fibrillated and old-age OA cartilage compared to young normal cartilage observed previously [3] may represent a combination of a ~10-15% decrease in tensile integrity with age [28] and an additional ~70% loss of tensile integrity with early degeneration observed in this study (Figure 3.2a). Additionally, the ~60% decrease in tensile ramp modulus and ~45% decrease in strength of mildly fibrillated cartilage compared to macroscopically normal cartilage observed by Kempson [29, 30] may have been slightly underestimated as values were averaged over lateral and (already-weak) medial sites.

Evidence of surface alteration in DGN and OA samples may indicate a partial loss or disruption of the superficial zone, directly affecting measures of superficial, middle, and deep layers. DGN and OA samples may have been adjacent to cartilage lesions and erosion but were selected from sites with mild degeneration localized to the superficial zone only. While the cartilage thickness indicates that these samples were without evidence of gross erosion, the thickness of OA samples may be a measure of combined partial cartilage erosion and tissue swelling. While measures made here are of the top, middle, and deep layers of cartilage for each group, they may no longer include an intact superficial zone.

An increase in water content is a hallmark of early osteoarthritis and is thought to be an indication of a weakened collagen network [9, 33]. The increase in water content can influence measures of matrix molecules when normalized to the wet weight of the tissue. Values obtained in this study for total collagen and GAG content agree with those found in previous studies [30, 34, 35, 53] for aged normal and mildly degenerate articular cartilage. In this study, an overall lower concentration of COL

and GAG per volume of swollen OA tissue was observed. However, there might not have been a net loss of these molecules from the matrix.

A similar effect was observed with the measure of cell density. The tissue swelling resulted in an apparent decrease in cell density in the superficial and middle layers of OA samples. While there may be a decrease in cell density per volume of tissue, there may not have been a net loss of cells from the tissue. In fibrillated tissue there may be regions of cell cloning and regions of hypo-cellularity associated with cell death [1, 41, 47]. Cell clusters are believed to form to compensate for cell loss and increase overall synthetic activity, but it is, as yet, unknown what effect their activity has on matrix anabolism [2]. It remains unclear if the decrease in cell density noted in the prior histological analyses is due to cell death or if the presence of empty lacunae is due to a histological artifact of cell loss [1]. Biochemical analyses of Gnd and aCT extracts showed ~10% of DNA in DGN and OA samples was in the extracellular space (data not shown). This was higher than that in NL samples (<2%, each $p<0.01$), but not site or depth-dependent ($p=0.4$-$0.8$). This indicates that a large proportion of measured DNA is no longer within cells but rather in the extracellular space, possibly due to cell death.

Collagen degradation may be an indication of chondrocyte activation in OA. The higher water content of OA samples is suggestive of a degraded collagen network [9, 33]. The higher amount of degraded collagen in DGN and OA samples may be indicative of some level of increased enzymatic activity resulting in cleavage and denaturation of the collagen network [16, 24]. The higher COL in aCT as well as the lower fluorescence ratio of DGN and OA samples compared to NL are both

suggestive of chondrocyte activation which may lead to collagen degradation [21] and synthesis of new molecules [18, 20, 44] including newly synthesized collagen not yet incorporated into the collagen network [26, 54]. The measure of COL in aCT in this study is an indirect measure of type II collagen degradation. Activated chondrocytes may undergo phenotypic changes resulting in the synthesis of collagen other than type II [20] which may be included in measures here. A downstream consequence of the altered synthesis and degradation of collagen molecules may be an inferior collagen network.

Chondrocyte activation could also result in alterations of GAG chains and aggrecan monomers not measured in this study. Chondroitin sulfate chains, for example, have been shown to have non-reducing terminal disaccharides with variable 4- and 6-sulfated and 4,6 disulfated residues depending on the degree of OA disease [45]. In addition, the size and number of chondroitin sulfate chains can decrease in degenerate articular cartilage [12, 23], which may be partially attributable to the selective proteolytic degradation of the GAG-rich region of aggrecan [17].

This study highlights changes in the tensile biomechanical properties of articular cartilage with the onset of early cartilage degeneration. It points to disruption of the articular surface and an altered collagen network as contributing factors to the progression of cartilage weakening in early degeneration (Figure 3.4). Tensile weakening with early degeneration occurs in superficial, middle, and deep layers (Figure 3.2c, d) such that surface wear may not be the sole contributor to the loss of tensile integrity. It may act in concert with collagen network remodeling, evidenced here as a higher amount of collagen degradation and lower amount of intrinsic

fluorescence in DGN and OA samples than NL samples (Figure 3.3c, d, e), to weaken the extracellular matrix. Additional work is needed to elucidate the mechanisms behind surface wear and collagen network remodeling in early cartilage degeneration and the sequence of events leading to overt cartilage fibrillation and osteoarthritis.

## 3.6 Acknowledgments

This work was supported by the Arthritis Foundation, the National Institutes of Health, and the National Science Foundation. The dissertation author (primary investigator) thanks the coauthors of the manuscript for their contributions: Won Bae, Michael Chen, William Bugbee, David Amiel, and Richard Coutts. We thank Eun Hee Han, Jennifer Hwang, Gregory Williams, and Van Wong, UCSD, La Jolla, CA for assistance with testing and analysis of a portion of samples for this study.

## 3.7 References


1. Aigner T, Hemmel M, Neureiter D, Gebhard PM, Zeiler G, Kirchner T, McKenna L: Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritic human articular knee cartilage. *Arthritis Rhuem* 44:1304-12, 2001.

2. Aigner T, Vornehm SI, Zeiler G, Dudhia J, von der Mark K, Bayliss MT: Suppression of cartilage matrix gene expression in upper zone chondrocytes of osteoarthritic cartilage. *Arthritis Rheum* 40:562-9, 1997.

3. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH: Tensile properties of human knee joint cartilage: I. influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus. *J Orthop Res* 4:379-92, 1986.

4. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH: Tensile properties of human knee joint cartilage: II. correlations between weight bearing and tissue pathology and the kinetics of swelling. *J Orthop Res* 5:173-86, 1987.

5. Bader DL, Kempson GE, Barrett AJ, Webb W: The effects of leucocyte elastase on the mechanical properties of adult human articular cartilage in tension. *Biochim Biophys Acta* 677:103-8, 1981.

6. Bae WC, Temple MM, Amiel D, Coutts RD, Niederauer GG, Sah RL: Indentation testing of human cartilage: sensitivity to articular surface degeneration. *Arthritis Rheum* 48:3382-94, 2003.

7. Bank RA, Bayliss MT, Lafeber FPJG, Maroudas A, TeKoppele JM: Ageing and zonal variation in post-translational modification of collagen in normal human articular cartilage. The age-related increase in non-enzymatic glycation affects biomechanical properties of cartilage. *Biochem J* 330:345-51, 1998.

8. Bank RA, Krikken M, Beekman B, Stoop R, Maroudas A, Lafeber FPJG, Te Koppele JM: A simplified measurement of degraded collagen in tissues: application in healthy, fibrillated and osteoarthritic cartilage. *Matrix Biol* 16:233-43, 1997.

9. Bank RA, Soudry M, Maroudas A, Mizrahi J, TeKoppele JM: The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. *Arthritis Rheum* 43:2202-10, 2000.

10. Bennett GA, Waine H, Bauer W. Changes in the knee joint at various ages with particular reference to the nature and development of degenerative joint disease. New York: The Commonwealth Fund; 1942.

11. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chan J, Van Wart H, Poole AR: Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* 99:1534-45, 1997.

12. Bollet AJ, Nance JL: Biochemical findings in normal and osteoarthritic articular cartilage. II: chondroitin surface concentration and chanin length, water, and ash content. *J Clin Invest* 45:1170-7, 1966.

13. Chen AC, Klisch SM, Bae WC, Temple MM, McGowan KB, Gratz KR, Schumacher BL, Sah RL: Mechanical characterization of native and tissue-engineered cartilage. In: *Cartilage and Osteoarthritis,* ed. by M Sabatini, DeCeuninck F, Pastoureau P, Humana Press, Totowa, NJ, 2004, 157-90.

14. Clark JM, Simonian PT: Scanning electron microscopy of "fibrillated" and "malacic" human articular cartilage: technical considerations. *Microsc Res Tech* 37:299-313, 1997.

15. Dean DD, Martel-Pelletier J, Pelletier JP, Howell DS, Woessner JFJ: Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. *J Clin Invest* 84:678-85, 1989.

16. Dodge GR, Poole AR: Immunohistochemical detection and immunochemical analysis of type II collagen degradation in human normal rheumatoid, and osteoarthritis articular cartilage and in explants of bovine articular cartilage cultured with interleukin 1. *J Clin Invest* 83:647-61, 1989.

17. Dudhia J, Davidson CM, Wells TM, Vynios DH, Hardingham TE, Bayliss MT: Age-related changes in the content of the C-terminal region of aggrecan in human articular cartilage. *Biochem J* 313:933-40, 1996.

18. Eyre DR: Collagen structure and function in articular cartilage: metabolic changes in the development of osteoarthritis. In: *Osteoarthritic Disorders,* ed. by KE Kuettner, Goldberg VM, American Academy of Orthopaedic Surgeons, Rosemont, IL, 1995, 219-27.

19. Farndale RW, Buttle DJ, Barrett AJ: Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 883:173-7, 1986.

20. Gibson G, Lin DL, Francki K, Caterson B, Foster B: Type X collagen is colocalized with a proteoglycan epitope to form distinct morphological structures in bovine growth cartilage. *Bone* 19:307-15, 1996.

21. Gibson GJ, Verner JJ, Nelson FR, Lin DL: Degradation of the cartilage collagen matrix associated with changes in chondrocytes in osteoarthrosis. Assessment by

loss of background fluroescence and immunodetection of matrix components. *J Orthop Res* 19:33-42, 2001.

22. Harrington IJ: A bioengineering analysis of force actions at the knee in normal and pathological gait. *Biomed Eng* 11:167-72, 1976.

23. Hjertquist SO, Wasteson A: The molecular weight of chondroitin sulfate from human articular cartilage: effects of age and osteoarthritis. *Calc Tiss Res* 10:31-7, 1972.

24. Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, Poole AR: Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. *J Clin Invest* 93:1722-32, 1994.

25. Hollander AP, Pidoux I, Reiner A, Rorabeck C, Bourne R, Poole AR: Damage to type II collagen in aging and osteoarthritis starts at the articular surface, orginates around chondrocytes, and extends into the cartilage with progressive degeneration. *J Clin Invest* 96:2859-69, 1995.

26. Hoyland JA, Thomas JT, Donn R, Marriott A, Ayad S, Boot-Handford RP, Grant ME, Freemont AJ: Distribution of type X collagen mRNA in normal and osteoarthritic human cartilage. *Bone Miner* 15:151-63, 1991.

27. Jackson DS, Cleary EG: The determination of collagen and elastin. *Methods Biochem Anal* 15:25-76, 1967.

28. Kempson GE: Relationship between the tensile properties of articular cartilage from the human knee and age. *Ann Rheum Dis* 41:508-11, 1982.

29. Kempson GE, Freeman MAR, Swanson SAV: Tensile properties of articular cartilage. *Nature* 220:1127-8, 1968.

30. Kempson GE, Muir H, Pollard C, Tuke M: The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. *Biochim Biophys Acta* 297:456-72, 1973.

31. Kempson GE, Tuke MA, Dingle JT, Barrett AJ, Horsfield PH: The effects of proteolytic enzymes on the mechanical properties of adult human articular cartilage. *Biochim Biophys Acta* 428:741-60, 1976.

32. Lawrence RC, Helmick CG, Arnett FC, Deyo RA, Felson DT, Giannini EH, Heyse SP, Hirsch R, Hochberg MC, Hunder GG, Liang MH, Pillemer SR, Steen VD, Wolfe F: Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. *Arthritis Rheum* 41:778-99, 1998.

33. Maroudas A: Balance between swelling pressure and collagen tension in normal and degenerate cartilage. *Nature* 260:808-9, 1976.

34. Maroudas A: Physico-chemical properties of articular cartilage. In: *Adult Articular Cartilage,* ed. by MAR Freeman, Pitman Medical, Tunbridge Wells, England, 1979, 215-90.

35. Maroudas A, Bayliss MT, Venn MF: Further studies on the composition of human femoral head cartilage. *Ann Rheum Dis* 39:514-23, 1980.

36. Maroudas A, Palla G, Gilav E: Racemization of aspartic acid in human articular cartilage. *Connect Tissue Res* 28:161-9, 1992.

37. Maroudas A, Ziv I, Weisman N, Venn M: Studies of hydration and swelling pressure in normal and osteoarthritic cartilage. *Biorheology* 22:159-69, 1985.

38. McGowan KB, Kurtis MS, Lottman LM, Watson D, Sah RL: Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen® and Hoechst 33258. *Osteoarthritis Cartilage* 10:580-7, 2002.

39. Meachim G: Light microscopy of Indian ink preparations of fibrillated cartilage. *Ann Rheum Dis* 31:457-64, 1972.

40. Meachim G, Emery IH: Quantitative aspects of patello-femoral cartilage fibrillation in Liverpool necropsies. *Ann Rheum Dis* 33:39-47, 1974.

41. Mitrovic D, Quintero M, Stankovic A, Ryckewaert A: Cell density of adult human femoral condylar articular cartilage. *Lab Invest* 49:309-16, 1983.

42. Morrison JB: The mechanics of the knee joint in relation to normal walking. *J Biomech* 3:51-61, 1970.

43. Muehleman C, Bareither D, Huch K, Cole AA, Kuettner KE: Prevalence of degenerative morphological changes in the joints of the lower extremity. *Osteoarthritis Cartilage* 5:23-37, 1997.

44. Plaas AHK, Sandy JD: Proteoglycan anabolism and catabolism in articular cartilage. In: *Osteoarthritic Disorders,* ed. by KE Kuettner, Goldberg VM, American Academy of Orthopaedic Surgeons, Rosemont, IL, 1995, 103-16.

45. Plaas AHK, West LA, Wong-Palms S, Nelson FR: Glycosaminoglycan sulfation in osteoarthritis: alterations at the non-reducing termini of chondroitin and dermatan sulfate. *J Biol Chem* 273:12642-9, 1998.

46. Praemer A, Furner S, Rice DP. Musculoskeletal conditions in the United States. Park Ridge, IL: American Academy of Orthopaedic Surgeons; 1999.

47. Quintero M, Mitrovic DR, Stankovic A, de Seze S, Miravet L, Ryckewaert A: Aspects cellulaires du vieillissement du cartilage articulaire. II. cartilage

condylien a surface fissuree preleve dans les genoux normaux et arthrosiques. *Revue du Rhumatisme* 51:445-9, 1984.

48. Rizkalla G, Reiner A, Bogoch E, Poole AR: Studies of the articular cartilage proteoglycan aggrecan in health and osteoarthritis: evidence for molecular heterogeneity and extensive molecular changes in disease. *J Clin Invest* 90:2268-77, 1992.

49. Sandy JD, Neame PJ, Boynton RE, Flannery CR: Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. *J Biol Chem* 266:8683-5, 1991.

50. Sokal RR, Rohlf FJ. Biometry. 3rd ed. New York: WH Freeman and Co.; 1995.

51. Stockwell RA: The cell density of human articular and costal cartilage. *J Anat* 101:753-63, 1967.

52. Towle CA, Hung HH, Bonassar LJ, Treadwell BV, Mangham DG: Detection of interleukin-1 in the cartilage of patients with osteoarthritis: a possible autocrine/paracrine role in pathogenesis. *Osteoarthritis Cartilage* 5:293-300, 1997.

53. Treppo S, Koepp H, Quan EC, Cole AA, Kuettner KE, Grodzinsky AJ: Comparison of biomechanical and biochemical properties of cartilage from human knee and ankle pairs. *J Orthop Res* 18:739-48, 2000.

54. von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Glückert K, Stöss H: Type X collagen synthesis in human osteoarthritic cartilage. *Arthritis Rheum* 35:806-10, 1992.

55. Webber C, Glant TT, Roughley PJ, Poole AR: The identification and characterization of two populations of aggregating proteoglycans of high buoyant density isolated from post-natal human articular cartilages of different ages. *Biochem J* 236:71-5, 1987.

56. Westacott CI, Whicher JT, Barnes IC, Thompson D, Swan AJ, Dieppe PA: Synovial fluid concentrations of five different cytokines in rheumatic diseases. *Ann Rheum Dis* 49:676-81, 1990.

57. Williamson AK, Chen AC, Masuda K, Thonar EJ-MA, Sah RL: Tensile mechanical properties of bovine articular cartilage: variations with growth and relationships to collagen network components. *J Orthop Res* 21:872-80, 2003.

58. Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 93:440-7, 1961.

59. Wood DD, Ihrie EJ, Dinarello CA, Cohen PL: Isolation of an interleukin-1-like factor from human joint effusions. *Arthritis Rheum* 26:975-83, 1983.

# CHAPTER 4

# DECREASED CELLULARITY IN THE SUPERFICIAL ZONE OF HUMAN ARTICULAR CARTILAGE IN EARLY DEGENERATION

## 4.1 Abstract

Objective. To determine and compare the depth-associated variation in cell density and organization of superficial and transitional zones of young normal, aged normal, and aged, mildly degenerate adult human articular cartilage using two-dimensional (2D) and three-dimensional (3D) imaging methods.

Methods. Macroscopically normal or mildly degenerate human articular cartilage of the medial femoral condyles from Young (21-39 yrs) and Aged (≥40 yrs) donors were characterized by India ink staining and then analyzed for cell density in 2D histological sections and in 3D *en face* views of cartilage cell nuclei obtained by confocal laser scanning microscopy.

Results. Cell density was markedly lower in aged degenerate cartilage than young normal cartilage in both vertical and *en face* views, and tended to be lower in aged normal than young normal cartilage when quantified in *en face* views. The lower

cell density in samples with mild degeneration was localized to regions near the articular surface and was paralleled by changes in cell spatial organization of the superficial zone in *en face* views, from large cell clusters to smaller clusters of cells.

Conclusions. The decreased cell density and altered spatial organization of chondrocytes of the superficial zone may result in large areas of matrix devoid of the capacity for matrix remodeling or repair. This may result in regions within the superficial zone of early degenerate cartilage prone to a loss of mechanical integrity. Further study is needed to elucidate the role of altered spatial organization on matrix homeostasis and cartilage weakening.

## 4.2 Introduction

Alteration of articular cartilage cellularity is believed to play a significant role in the progression of cartilage degeneration. Through cell counting in histological sections, the cell density of macroscopically normal human articular cartilage from a macroscopically normal joint decreases with age [24, 30], but may be dependent on anatomical location and depth from the articular surface [35]. Also, through cell counting, macroscopically normal cartilage from an osteoarthritic (OA) joint appears to have a lower cell density than that from a normal joint in the superficial, transitional, and radial zones for donors under the age of 60 [24, 31]. In older donors the cell density of articular cartilage is about the same in all zones for normal and OA joints [24, 31]. A diminished cell density with age and with early cartilage degeneration may predispose cartilage to a loss of mechanical integrity, with remaining cells being unable to maintain matrix homeostasis.

However, it has been suggested that histological preparation and analysis may introduce error into the quantitation of cell density. Cells may be lost during histological preparation, resulting in the presence of empty lacunae [1]. However, stereological approaches are needed to estimate cell density more robustly and to account for variations in cell shape [12, 15, 27, 28, 38]. In contrast, confocal microscopy allows the viewing of objects *in situ* and in three dimensions (3D) in relatively large volumes of tissue. This method has been used for qualitative analyses [1], but can also be analyzed for quantitative measures of cell density [16]. Analysis of the density and 3D organization of chondrocytes, especially of the superficial zone

where cells have a flattened morphology [33], may yield a precise estimate and improved understanding of the cellularity of cartilage in degeneration.

Degeneration of articular cartilage leading to osteoarthritic erosion has a predilection for certain sites in certain joints. Early degenerative changes are more prevalent in the medial femoral condyle (MFC) than the lateral femoral condyle (LFC) [5]. When degenerative changes are present, they are usually more severe in the MFC than the LFC [26] and include a decrease in cartilage thickness of the MFC, but not the LFC of patients with knee OA [7]. In addition, the medial compartment of the knee is subjected to higher joint pressures than the lateral compartment during the stance phase of normal gait [14, 25] and has a lower indentation stiffness than does that of the LFC [4, 20], properties which may contribute to the progressive early degenerative changes in the MFC. Thus, analysis of cellularity would be particularly interesting in macroscopically normal and early degenerate samples of the MFC.

The objective of this study was to determine and compare the depth-associated variation in cell density and organization of superficial and transitional zones of young normal, aged normal, and aged, mildly degenerate adult human articular cartilage of the MFC using two-dimensional (2D) histology and 3D confocal microscopy.

## 4.3 Materials and Methods

**Sample selection and preparation.** Two related studies were performed. For both studies, samples from the anterior region of the MFC were selected for analysis, based on donor age and limited cartilage degeneration. For Study I, a total of 34

samples (donors) were Young (21-40 yrs old) or Aged (>40 yrs old), and had a macroscopic articular surface appearance that was either smooth (NL, grade 0) or mildly fibrillated (DGN, grade 1), according to the modified Collins grading scheme [10, 26]. The distribution of samples (Table 4.1) was approximately even between male ($n$=4-7) and female ($n$=3-5) donors for Young NL, Aged NL, and Aged DGN groups, with a body mass index (BMI) that was similar between these three experimental groups. For Study II, a total of 16 additional samples were isolated. Characteristics of these samples are described in Table 4.2.

Samples were initially harvested in the form of 10-mm diameter osteochondral cores with a notch placed in the posterior-most edge of the core in order to maintain orientation. Samples were soaked in phosphate-buffered saline (PBS) with proteinase inhibitors (PI; 1 mM PMSF, 2 mM EDTA, 5 mM benzamidine-HCl, and 10 mM N-ethylmaleimide) [13] at 4°C for one hour and then stored at –70°C until use. Samples were subsequently thawed in PBS+PI for 15 minutes at room temperature, and analyzed for reflectance score after India ink staining and for cartilage thickness.

| | | Medial Femoral Condyle | | |
|---|---|---|---|---|
| | | Young NL | Aged NL | Aged DGN |
| Age (yr) | Female | 33±2 (5) | 60±5 (7) | 67±9 (3) |
| | Male | 34±2 (5) | 58±3 (11) | 70±8 (3) |
| | Female+Male | 33±1 (10) | 59±3 (18) | 69±5 (6) |
| BMI ($kg/m^2$) | Female | 28±4 | 19±1 | 23±5 |
| | Male | 25±2 | 24±1 | 24±4 |
| | Female+Male | 27±2 | 22±1 | 24±3 |

**Table 4.1:** Sample characteristics for Study I, on 2D depth-varying cell density. Age reported and body mass index reported as mean ± SEM for (n) samples from human female and male donors of osteochondral cores from the medial femoral condyle.

| | | Medial Femoral Condyle | | |
|---|---|---|---|---|
| | | Young NL | Aged NL | Aged DGN |
| Age (yr) | Female | 29±8 (2) | 68 (1) | 69±3 (3) |
| | Male | 32±3 (4) | 67±3 (4) | 71±2 (2) |
| | Female+Male | 31±3 (6) | 67±1 (5) | 69±2 (5) |
| BMI ($kg/m^2$) | Female | 25 | 28 | 21±2 |
| | Male | 23±5 | 24±1 | 23±2 |
| | Female+Male | 24±3 | 25±1 | 22±2 |

**Table 4.2:** Sample characteristics for Study II, on 3D depth-varying cell density. Age reported and body mass index reported as mean ± SEM for (n) samples from human female and male donors of osteochondral cores from the medial femoral condyle.

**India ink staining and image analysis.** The articular surface of each osteochondral core was stained with India ink, imaged, and analyzed to obtain a calibrated light reflectance score, as described previously [4, 8]. A reflectance score of 1 indicates relatively high reflectance, typical of normal cartilage, whereas a score of 0 indicates relatively low reflectance, typical of cartilage that is heavily stained with ink.

**Cartilage thickness measurement.** The cartilage thickness was determined from digital images taken at six equally-spaced points about the circumference of the osteochondral core [4]. Thickness measurements from the six images were averaged for each sample.

**Study I: 2D Depth-Varying Cell Density and Histopathology of Human Articular Cartilage.** In this study, a central region of each core was isolated and analyzed for a histopathological index of degradation using methods described previously [4, 34]. Briefly, 6-μm thick sections of tissue were obtained, stained with SafraninO-fast green, and imaged using transmitted light microscopy at magnifications of 4× and 10× (field of views of 2.5 × 3.9 $mm^2$ and 1.0 ×1.5 $mm^2$, respectively). The 4× images were analyzed to obtain a histopathology score (scale 0-15), where a low score corresponded to cartilage without evidence of gross macroscopic degeneration, surface irregularities, vertical clefts, transverse clefts, cloning, hypocellularity, or glycosaminoglycan loss, and a relatively high score corresponded to degenerate cartilage exhibiting these histopathological indices [4, 34]. The 10× images were analyzed to determine the area density of cells (#/$mm^2$) as a function of relative depth from the articular surface towards the tidemark. The

articular surface and tidemark were digitized, and a best-fit line to each of these landmarks was determined. The height of the articular cartilage was determined as the average distance between these lines. For the region from 0% (surface) to 50% (middle zone) of the cartilage thickness, cells were identified based on visibility of the cell membrane, localized manually, counted within each of 5 successive and equally-sized (10% thickness) bins, and normalized to bin area to determine cell density in each depth bin. Cells that were above the best-fit line at the articular surface, because of the roughness of the articular surface, were included in the 0-10% bin. Control studies indicated that this method of cell counting had minimal intra- and interobserver variability among four different observers (<9%).

**Study II: 3D Cell Density and Organization in the Superficial Zone of Human Articular Cartilage.** In this study, a portion of each osteochondral core was fixed in 4% paraformaldehyde in PBS, rinsed six times with PBS over 1 hr, and stained with a 1:200 dilution of PicoGreen® (dsDNA quantification reagent, Molecular Probes, Eugene, OR) over 24hrs at 4°C. Prior to imaging, samples were rinsed in PBS for 20 min. *In situ* images of the labeled cell nuclei were acquired with a confocal scanning laser microscope (MRC 1024, Bio-Rad Laboratories, Hercules, CA), using a 20X (NA 0.75) dry objective, an excitation wavelength of 488nm from an Ar/Kr laser, and an emission filter of >500nm, with detector gain and black level adjusted to measure a full dynamic range of intensities and iris size to maximize axial resolution. Cartilage was imaged *en face* in four separate $(545\mu m)^2$ areas of each sample, each to a depth of ~80μm at 0.75μm intervals, using LaserSharp Version 3.2 Acquisition and

Analysis Software (Bio-Rad). Images were imported into Matlab 7.0 (The MathWorks, Inc., Natick, MA) and processed to correct for depth-dependent attenuation of signal intensity [16]. For the air objective used, a factor of 1.33 was used to correct for depth alteration [6]. Cell edges were enhanced and background blur was reduced by convolution with a "Mexican Hat" filter [32]. Cell nuclei were identified by segmenting images at a threshold based on object count sensitivity, and localized based on their centroid [16]. The articular surface was defined as the plane of best fit through the 9 most superficial cells in each image stack. Cell nuclei were counted in, and normalized to, the tissue volumes included in a depth range of 0-25, 25-50, and 50-75μm from the articular surface, with 5μm at each edge excluded to eliminate edge effects. Data from the four regions of a sample were averaged to obtain representative measures for each sample in order to minimize error due to local variation of chondrocyte density.

**Statistics.** The effect of age/degeneration grouping (Young NL, Aged NL, Aged DGN) on thickness and reflectance score was assessed by ANOVA with Study (I and II) considered a random variable. When experimental group had an effect, post-hoc Tukey tests were performed. The effect of experimental group on the semi-quantitative histopathology score in Study I was assessed by the Kruskal-Wallis test, and differences between groups were assessed with Dunn's test. The effects of experimental group and (relative) depth on cell density were assessed by repeated measures ANOVA with experimental group as the main factor and (relative) depth (Study I: 0-10%, 10-20%, …, and 40-50%, and Study II: 0-25, 25-50, and 50-75μm)

as a repeated factor. When experimental group had an effect, planned comparisons were made between groups for each depth. All data are presented as mean ± SEM.

## 4.4 Results

The reflectance scores after India ink staining and cartilage thickness measurements were indicative of the MFC samples having an increasingly roughened articular surface with normal aging and with degeneration. After India ink staining, mild surface alterations were typically observed in Young NL, Aged NL, and Aged DGN samples (Figure 4.1). Surface striations were visible in Young NL and Aged NL samples, being generally more extensive in the latter, and a more mottled surface appearance was typical of Aged DGN samples. The qualitative observations were quantified as differences in the mean (Figure 4.2A) and the variance (Figure 4.2B) of the reflectance score. Aged NL and Aged DGN samples each tended to have lower mean reflectance scores than Young NL samples (each $p<0.005$), while the variance of reflectance score was similarly high for all three groups in these studies ($p=0.1$). Despite these variations discernible at the surface, the cartilage thickness (Figure 4.2C) was similar between experimental groups ($p=1.0$).

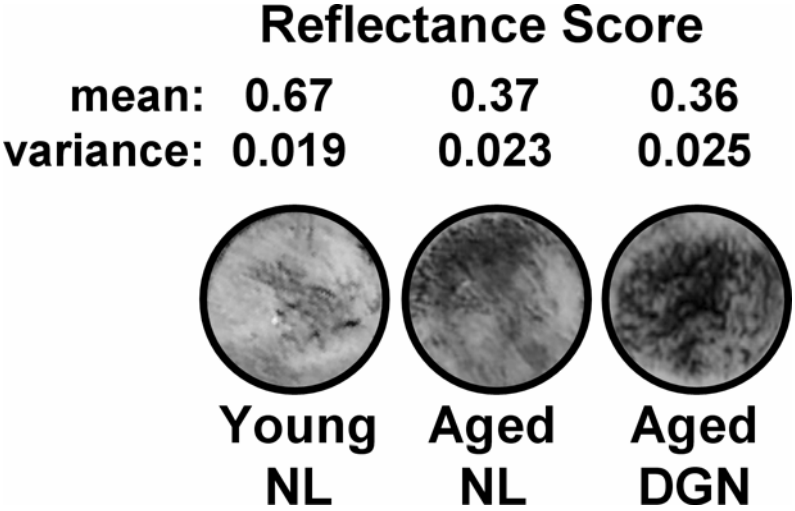



**Figure 4.1:** Representative images of India ink-stained articular cartilage surfaces. Osteochondral cores, 10mm in diameter, were obtained from the medial femoral condyle of Young (21-40 yrs old) and Aged (>40 yrs old) adult human donors and macroscopically graded as normal (NL) or mildly degenerate (DGN). The cartilage surface was stained with ink, digitally imaged, and analyzed for a reflectance score. Samples shown had the median reflectance score for their group, and the mean and variance of reflectance scores for the individual sample are shown.

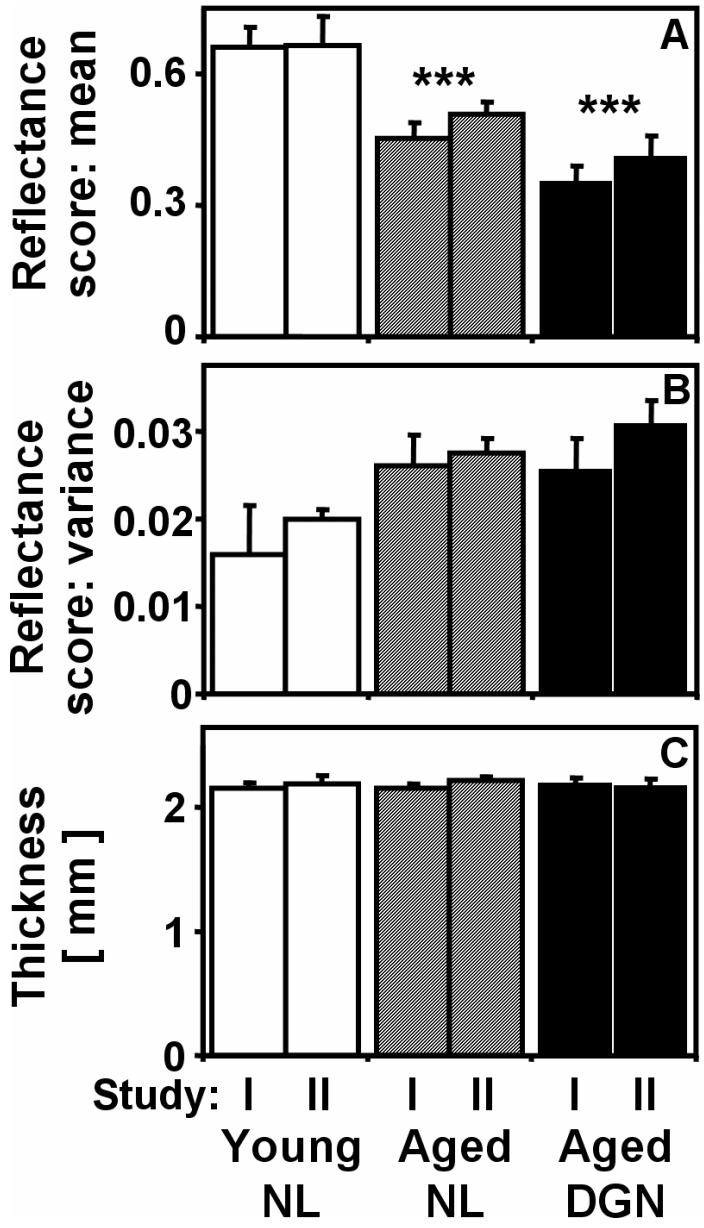



**Figure 4.2:** Structural properties of samples described in Tables 4.1 and 4.2. Samples were analyzed for the mean (**A**) and variance (**B**) of the reflectance score after ink-staining of the articular surface and for cartilage thickness (**C**). ***p<0.005 versus Young NL.

**Study I: 2D Depth-Varying Cell Density and Histopathology of Human Articular Cartilage.** Consistent with the pattern of change in reflectance score and images of India ink-stained cartilage surfaces, the vertical views of cartilage showed patterns of mild surface roughness in YNL and ANL samples (Figure 4.3A, B), and, consistent disruption of the surface in ADGN samples (Figure 4.3C). Correspondingly, the histopathology score (Figure 4.4A) was lowest in YNL samples, slightly higher in ANL samples ($p<0.05$), and higher in ADGN samples than either the ANL ($p<0.05$) or YNL samples ($p<0.001$). The increases in histopathology score were generally attributable to the surface irregularity (Figure 4.4B), which was higher in both ANL ($p<0.05$) and ADGN samples ($p<0.01$) than in YNL samples. Thus, cartilage surfaces of the MFC showed mild age-associated surface alteration and marked surface alteration with the onset of early cartilage degeneration.

Variations in cell density occurred in a manner that was dependent on experimental group and depth from the articular surface (Figure 4.5). The cell density generally decreased with depth from the articular surface (each $p<0.005$), but in a manner that was distinct for experimental group (interaction $p<0.005$). In particular, in the MFC, the cell density was ~48% higher in YNL samples ($p<0.005$) and ~50% higher in ANL samples ($p<0.005$) at the articular surface (0-10%) than in the middle zone (40-50%). However, the cell density was not depth-dependent in ADGN samples ($p=0.4$). In particular, the cell density at the 0-10% depth was lower in ADGN samples than that of either YNL (each $p<0.025$) or ANL samples (each $p<0.025$). At depths between 10 and 50%, the cell density was indistinguishable between experimental groups ($p=0.1$-$1.0$). The pattern of variation with age and degeneration in cell density

of the MFC, after 2D cell counting in vertical histological sections, indicates that changes occur with early degeneration, rather than with age, and are localized to the articular surface.

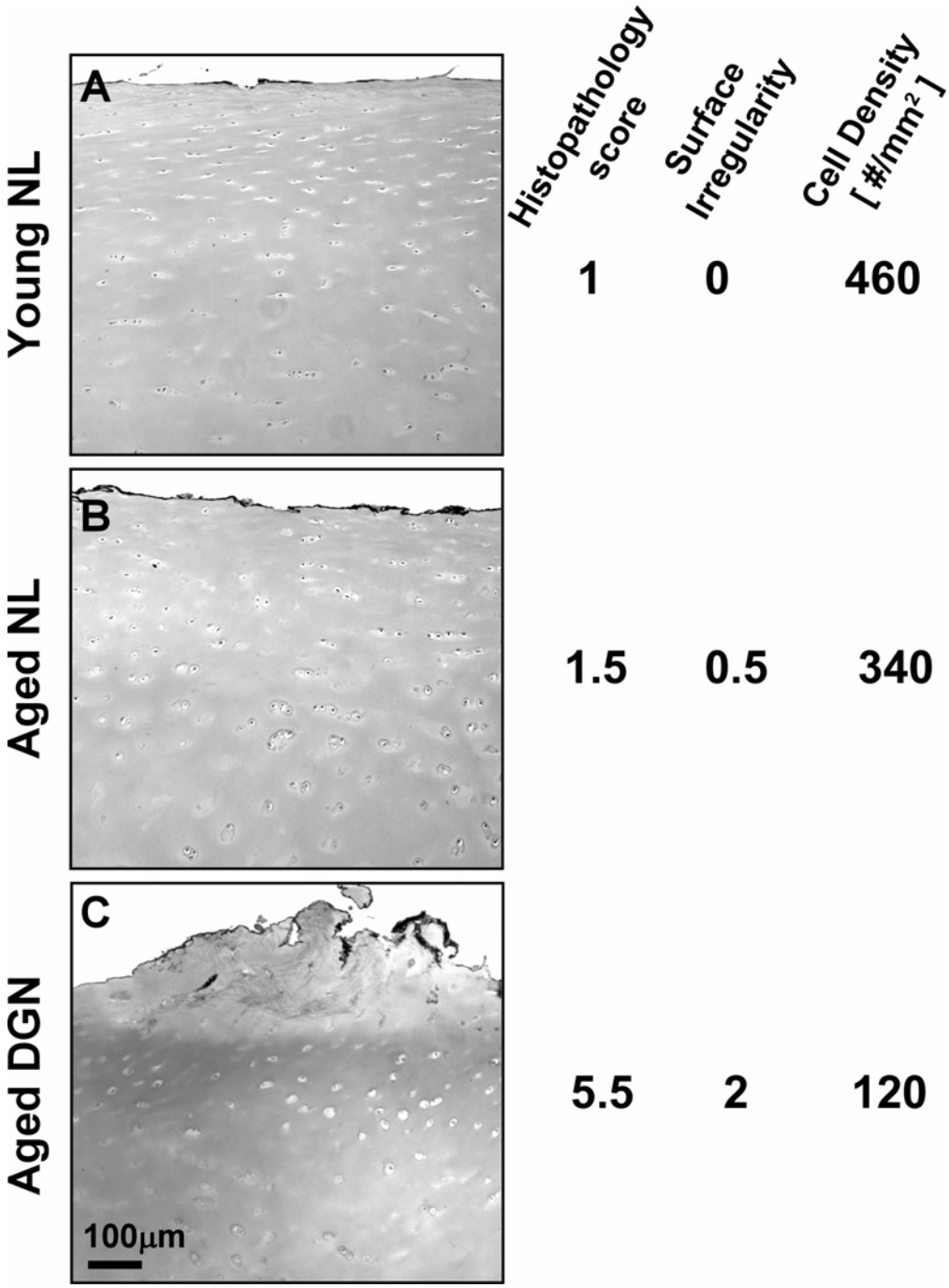



**Figure 4.3:** Representative micrographs of histological sections (vertical cut) of samples from Study I (Table 4.1). Sections were analyzed for a histopathology score (on a scale from 0, normal, to 15, degenerate), including surface irregularity (scale from 0 to 2), and cell density (in #/mm$^2$). Images shown had the median cell density for Young NL (**A**), Aged NL (**B**), and Aged DGN (**C**) groups.

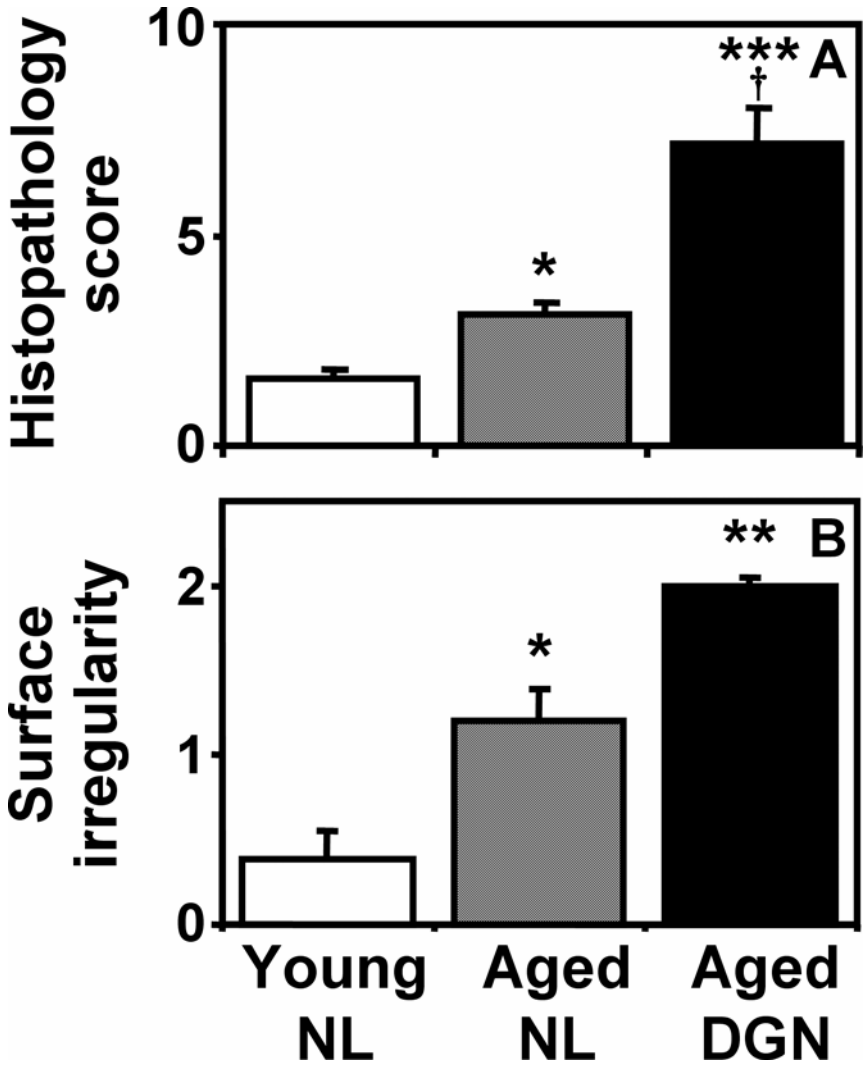



**Figure 4.4:** Structural properties of samples from Study I (Table 4.1). Samples were analyzed for a histopathology score (**A**) and surface irregularity (**B**). *p<0.05, **p<0.01, ***p<0.005 versus Young NL, † p<0.05 versus Aged NL.

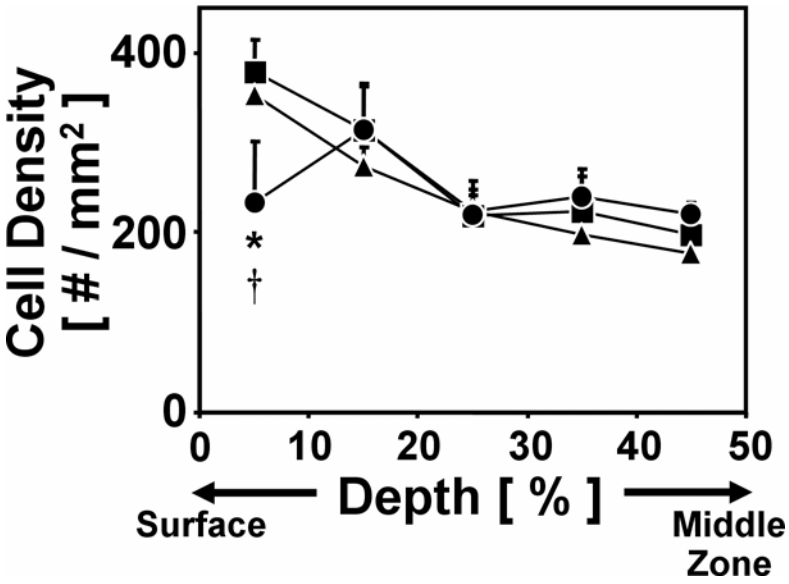



**Figure 4.5:** Cellularity of samples from Study I (Table 4.1). Samples were analyzed for cellularity (cell number per area) in tissue regions indicated, at a depth between 0% (surface) and 50% (middle zone) of the cartilage thickness. Young NL (■), Aged NL (▲), and Aged DGN (●) samples. *$p<0.05$ versus Young NL samples, †$p<0.05$ versus Aged NL samples.

**Study II: 3D Cell Density and Organization in the Superficial Zone of Human Articular Cartilage.** The density and spatial organization of chondrocytes appeared qualitatively altered in ADGN samples, compared to YNL and ANL samples. In 2D projections of the top 25μm, relatively large clusters of cell nuclei were evident in YNL (Figure 4.6A) and ANL (Figure 4.6B) samples, and relatively smaller clusters were present in ADGN samples (Figure 4.6C). The distinct appearance of YNL and ANL samples compared to ADGN samples was reflected in the quantitative analysis of 3D cell density.

The 3D-determined cell density tended to vary with experimental group ($p=0.09$) and varied significantly with depth ($p<0.005$). Cell density was highest in YNL samples, slightly lower in ANL samples and markedly lower in ADGN samples. In YNL and ANL samples, the cell density was highest in the 0-25μm region, being 35% and 34% higher, respectively, than in the 50-75μm region (each $p<0.05$, Figure 4.7). The variation in cell density with group was most evident in the 0-25μm region, where the cell density was highest in YNL samples, showing a trend of being lower in ANL samples (by 21%, $p=0.2$), and being markedly lower in the ADGN samples (by 43%, $p<0.05$). There was no significant variation in cell density in the 25-50μm and 50-75μm regions ($p=0.2$-$0.9$).

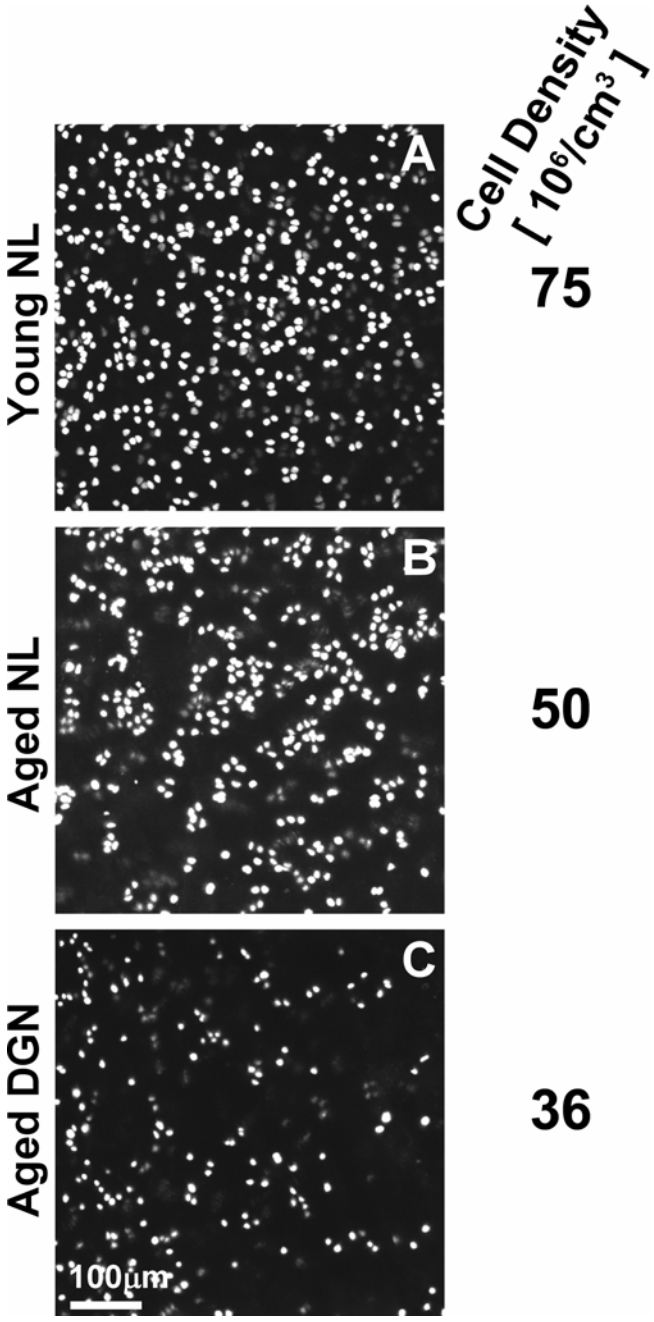



**Figure 4.6:** Representative *en face* views of cell nuclei of samples from Study II (Table 4.2). *En face* views were obtained by confocal laser scanning microscopy of fluorescently-stained samples and reducing 3D data from the top 25µm of each sample to a 2D projection. Data were also analyzed for cell density. Images, and accompanying values, are shown for samples with the median cell density from Young NL (**A**), Aged NL (**B**), and Aged DGN (**C**) groups.

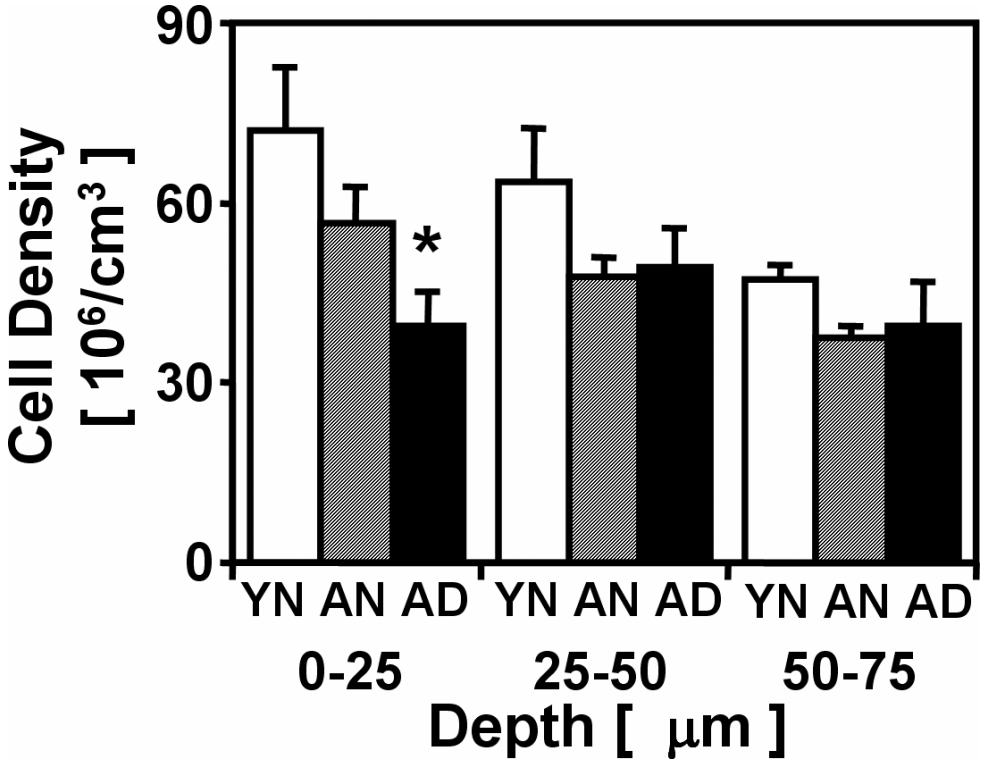



**Figure 4.7:** Cell number per volume of samples from Study II (Table 4.2). Osteochondral cores from Young Normal (YN), Aged Normal (AN) and Aged Degenerate (AD) groups were analyzed as shown in Figure 4.6. *$p<0.05$ versus YN.

## 4.5 Discussion

These studies identified alterations in the cell density of the superficial zone of adult human knee articular cartilage with age and early degeneration. In the samples studied, the extent of articular cartilage surface disruption, being very mild in YNL samples, slightly more in ANL samples, and moderate in ADGN samples, was confirmed by standard histopathological grading (Figures 4.3 and 4.4) as well as staining with India ink (Figure 4.1) as quantified by image analysis of reflectance (Figures 4.2A and B). The similarity in tissue thickness (Figure 4.2C) provided further evidence for the appropriateness of the samples selected. Compared to YNL samples, there was a mild decrease in cell density of ~25% in ANL samples, only observed in the top ~25μm of *en face* views (Figures 4.6 and 4.7), and a marked decrease in cell density of ~43% in ADGN samples, observable in vertical views (Figures 4.3 and 4.5) and localized to the top ~50μm by *en face* analysis (Figure 4.7). The pattern of change in cell density paralleled changes in the cellular organization observable in *en face* (Figure 4.6) views of articular cartilage. These results provide definitive evidence for a decrease in the cellularity of human articular cartilage of the MFC, associated with mild deterioration at the articular surface.

These studies focused on the superficial zone of articular cartilage because of its importance to the tensile integrity of cartilage [18] and its sensitivity to age [17] and degeneration [2, 3, 22]. The confocal laser scanning microscopy (CLSM) was thus targeted to this region by *en face* analysis at the articular surface. Using CLSM, chondrocytes in small tissue samples could be viewed *in situ*, without the need for

cartilage sectioning or removal from the subchondral bone. This minimizes tissue manipulation and eliminates the potential for artifactual loss of cells and cell nuclei due to histological processing [1]. With PicoGreen® fluorescence staining of cell nuclei, CLSM images could be captured up to a depth of ~100μm, similar to previous studies [37].

As with any study of human tissue, there are possible limitations due to sample selection. Normal-appearing cartilage in osteoarthritic joints can have elevated levels of matrix degrading enzymes [11] which may indicate abnormal cellular function and may have an unknown effect on cell density and organization. To minimize possible confounding effects of extensive joint deterioration, the macroscopically normal samples of these studies were obtained from donors without predisposing conditions for joint disorders and displayed no evidence of cartilage erosion in the femoral compartment. And, samples that were considered degenerate had surfaces that displayed mild fibrillation that was localized to the superficial zone. As confirmation of the sample selection, there were no fissures extending into the radial zone which have been associated with formation of brood capsules or pathological cell clustering [19, 23, 33]. Thus, the MFC samples studied are those from a human population that has undergone normal aging without development of osteoarthritis.

Further variation in cell density and spatial organization could be introduced depending on anatomical location. Cell density and organization may vary depending on the joint [33] as well as sites within a joint. This may be due to variations in loading patterns across joints [24]. Samples of these studies were selected consistently from the anterior MFC, the anterior-posterior orientation was maintained, and, in the

study of 3D cell density, multiple measurements were made within each sample to minimize the effects of site variation.

The findings by 2D analysis of cell density is consistent with previous studies of cell density in human articular cartilage. The depth-dependence of cell density determined by 2D analysis agrees with previous findings for macroscopically normal [23, 24, 30, 35] as well as mildly degenerate cartilage [24, 31].

Analysis of cell density by 3D *en face* analysis confirmed definitely the age- and degeneration-associated variations in cell density, and also gave more detail about these changes in the superficial zone. The direct 3D determination of decreased cell density with early degeneration is consistent with the 2D results, localizing changes in the top 50 μm. Whether alterations in cell density extend down further remain to be determined. In addition, the 3D analysis gave a strong trend for a decrease in cell density in the Aged NL samples in the most superficial 0-25μm layer, suggesting a progressive alteration starting from the articular surface. The absolute estimate of cell density from 3D analysis is, as expected, slightly higher than estimates of cell density by biochemistry [21] and stereological [15, 28] analysis, since the 3D analysis in the current study was localized to the top 75μm whereas the other studies examined regions of tissue extending deeper, and since cell density decreases steadily with distance from the articular surface.

It is the combined decrease in cell density and concurrent alteration of the spatial organization of cells which may result in the progression from early cartilage degeneration to osteoarthritis. The smaller cell clusters in ADGN samples may yield

an interterritorial region where matrix turnover is reduced [29] and the mechanical integrity of the cartilage matrix is compromised.

The observed cell organization is generally consistent with that found in other studies. The cell clusters organized parallel to the articular surface noted in *en face* views (Figure 4.2B) was similar to that reported previously for young adult human articular cartilage [33, 36]. This cell organization was discernible in *en face* views rather than vertical views of cartilage, because tangentially-arranged clusters appear in cross-section as an approximately linear (horizontal) arrangement of only one to three cells. Some of the hypocellularity determined in *en face* views could result form undulation of the roughened cartilage surface, resulting in imaging of volumes where tissue matrix was not present. However, this appeared unlikely to be a major effect since samples were imaged with the articular surface resting face down against a glass surface, causing a slight compaction of protruding tissue.

The analysis of cell density presented in this study could be extended by quantitatively characterizing the spatial organization of chondrocytes in 3D. The complex morphology and organization of chondrocytes in macroscopically normal cartilage may reflect a characteristic organization necessary for maintaining matrix homeostasis. Quantitative assessment of the 3D organization of chondrocytes may lead to further understanding of the role of this organization in aging and degeneration. In addition, further study is needed to fully elucidate the alterations in cell density and spatial organization of cells in the radial and deep zones with age and degeneration, and new tools for analyzing these characteristics in 3D [16] could provide valuable new insights.

This decreased cell density and altered spatial organization of chondrocytes in mildly degenerate cartilage may predispose such tissue to mechanical failure. At this early stage of cartilage degeneration, the corresponding cell clustering in the superficial zone may be insufficient to make up for the loss of cells, contributing to the progressive degeneration of that region. Fewer cells become responsible for increasingly large areas of matrix, reducing turnover of matrix far from the cells and diminishing the capacity for cartilage repair. In addition, an altered pattern of cell communication [9], or complete loss of cell contact, may result in an alteration of matrix proteins produced by the cells and creation of an inferior extracellular matrix. Mildly degenerate cartilage may deteriorate further and progress to osteoarthritic disease.

## 4.6 Acknowledgments

This work was supported by the Arthritis Foundation, the National Institutes of Health, and the National Science Foundation. The dissertation author (primary investigator) thanks the coauthors of the manuscript for their contributions: Won Bae, Kyle Jadin, Michael Chen, and Jeffrey Price. We thank Omar Bissar, Steven Moseley, and Scott Tcheng for their assistance with imaging and analysis of samples for this study.

## 4.7 References


1. Aigner T, Hemmel M, Neureiter D, Gebhard PM, Zeiler G, Kirchner T, McKenna L: Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritic human articular knee cartilage. *Arthritis Rhuem* 44:1304-12, 2001.

2. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH: Tensile properties of human knee joint cartilage: I. influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus. *J Orthop Res* 4:379-92, 1986.

3. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH: Tensile properties of human knee joint cartilage: II. correlations between weight bearing and tissue pathology and the kinetics of swelling. *J Orthop Res* 5:173-86, 1987.

4. Bae WC, Temple MM, Amiel D, Coutts RD, Niederauer GG, Sah RL: Indentation testing of human cartilage: sensitivity to articular surface degeneration. *Arthritis Rheum* 48:3382-94, 2003.

5. Bennett GA, Waine H, Bauer W. Changes in the knee joint at various ages with particular reference to the nature and development of degenerative joint disease. New York: The Commonwealth Fund; 1942.

6. Bucher D, Scholz M, Stetter M, Obermayer K, Pfluger HJ: Correction methods for three-dimensional reconstructions from confocal images: I. Tissue shrinking and axial scaling. *J Neurosci Methods* 100:135-43, 2000.

7. Buckland-Wright JC, Macfarlane DG, Lynch JA, Jasani MK, Bradshaw CR: Joint space width measures cartilage thickness in osteoarthritis of the knee: high resolution plain film and double contrast macroradiographic investigation. *Ann Rheum Dis* 54:263-8, 1995.

8. Chang DG, Iverson EP, Schinagl RM, Sonoda M, Amiel D, Coutts RD, Sah RL: Quantitation and localization of cartilage degeneration following the induction of osteoarthritis in the rabbit knee. *Osteoarthritis Cartilage* 5:357-72, 1997.

9. Chi SS, Rattner JB, Matyas JR: Communication between paired chondrocytes in the superficial zone of articular cartilage. *J Anat* 205:363-70, 2004.

10. Collins DH. The pathology of articular and spinal disease. London: Arnold; 1949.

11. Dean DD, Martel-Pelletier J, Pelletier JP, Howell DS, Woessner JFJ: Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. *J Clin Invest* 84:678-85, 1989.

12. Eggli PS, Hunziker EB, Schenk RK: Quantitation of structural features characterizing weight- and less-weight-bearing regions in articular cartilage: a

stereological analysis of medial femoral condyles in young adult rabbits. *Anat Rec* 222:217-27, 1988.

13. Frank EH, Grodzinsky AJ, Koob TJ, Eyre DR: Streaming potentials: a sensitive index of enzymatic degradation in articular cartilage. *J Orthop Res* 5:497-508, 1987.

14. Harrington IJ: A bioengineering analysis of force actions at the knee in normal and pathological gait. *Biomed Eng* 11:167-72, 1976.

15. Hunziker EB, Quinn TM, Hauselmann HJ: Quantitative structural organization of normal adult human articular cartilage. *Osteoarthritis Cartilage* 10:564-72, 2002.

16. Jadin KD, Wong BL, Bae WC, Li KW, Williamson AK, Schumacher BL, Price JH, Sah RL: Depth-varying density and organization of chondrocyte in immature and mature bovine articular cartilage assessed by 3-D imaging and analysis. *J Histochem Cytochem* (In press), 2005.

17. Kempson GE: Relationship between the tensile properties of articular cartilage from the human knee and age. *Ann Rheum Dis* 41:508-11, 1982.

18. Kempson GE, Muir H, Pollard C, Tuke M: The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. *Biochim Biophys Acta* 297:456-72, 1973.

19. Kouri JB, Jimenez SA, Quintero M, Chico A: Ultrastructural study of chondrocytes from fibrillated and non-fibrillated human osteoarthritic cartilage. *Osteoarthritis Cartilage* 4:111-25, 1996.

20. Lyyra T, Kiviranta I, Vaatainen U, Helminen HJ, Jurvelin JS: In vivo characterization of indentation stiffness of articular cartilage in the normal human knee. *J Biomed Mater Res* 48:482-7, 1999.

21. McGowan KB, Kurtis MS, Lottman LM, Watson D, Sah RL: Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen® and Hoechst 33258. *Osteoarthritis Cartilage* 10:580-7, 2002.

22. Meachim G: Light microscopy of Indian ink preparations of fibrillated cartilage. *Ann Rheum Dis* 31:457-64, 1972.

23. Meachim G, Collins DH: Cell count of normal and osteoarthritic articular cartilage in relation to the uptake of sulphate (35SO4) in vitro. *Ann Rheum Dis* 21:45, 1962.

24. Mitrovic D, Quintero M, Stankovic A, Ryckewaert A: Cell density of adult human femoral condylar articular cartilage. *Lab Invest* 49:309-16, 1983.

25. Morrison JB: The mechanics of the knee joint in relation to normal walking. *J Biomech* 3:51-61, 1970.

26. Muehleman C, Bareither D, Huch K, Cole AA, Kuettner KE: Prevalence of degenerative morphological changes in the joints of the lower extremity. *Osteoarthritis Cartilage* 5:23-37, 1997.

27. Paukkonen K, Selkainaho K, Jurvelin J, Helminen HJ: Morphometry of articular cartilage: a stereological method using light microscopy. *Anat Rec* 210:675-82, 1984.

28. Quinn TM, Hunziker EB, Hauselmann HJ: Variation of cell and matrix morphologies in articular cartilage among locations in the adult human knee. *Osteoarthritis Cartilage* 13:672-8, 2005.

29. Quinn TM, Maung AA, Grodzinsky AJ, Hunziker EB, Sandy JD: Physical and biological regulation of proteoglycan turnover around chondrocytes in cartilage explants. Implications for tissue degradation and repair. *Ann NY Acad Sci* 878:420-41, 1999.

30. Quintero M, Mitrovic DR, Stankovic A, de Seze S, Miravet L, Ryckewaert A: Aspects cellulaires du vieillissement du cartilage articulaire. I. cartilage condylien a surface normale, preleve dans les genoux normaux. *Revue du Rhumatisme* 51:375-9, 1984.

31. Quintero M, Mitrovic DR, Stankovic A, de Seze S, Miravet L, Ryckewaert A: Aspects cellulaires du vieillissement du cartilage articulaire. II. cartilage condylien a surface fissuree preleve dans les genoux normaux et arthrosiques. *Revue du Rhumatisme* 51:445-9, 1984.

32. Russ C. The Image Processing Handbook. 3rd ed. Boca Raton: CRC; 1999.

33. Schumacher BL, Su J-L, Lindley KM, Kuettner KE, Cole AA: Horizontally oriented clusters of multiple chondrons in the superficial zone of ankle, but not knee articular cartilage. *Anat Rec* 266:241-8, 2002.

34. Shapiro F, Glimcher MJ: Induction of osteoarthrosis in the rabbit knee joint. Histologic changes following menisectomy and meniscal lesions. *Clin Orthop* 147:287-95, 1980.

35. Stockwell RA: The cell density of human articular and costal cartilage. *J Anat* 101:753-63, 1967.

36. Stockwell RA, Meachim G: The chondrocytes. In: *Adult Articular Cartilage,* ed. by MAR Freeman, Pitman Medical, Tunbridge Wells, England, 1979, 69-144.

37. Wong B, Wallace V, Coleno M, Benton H, Tromberg B: Two-Photon Excitation Laser Scanning Microscopy of Human, Porcine, and Rabbit Nasal Septal Cartilage. *Tissue Engineering* 7:599-606, 2001.

38. Wong M, Wuethrich P, Eggli P, Hunziker E: Zone-specific cell biosynthetic activity in mature bovine articular cartilage: a new method using confocal microscopic stereology and quantitative autoradiography. *J Orthop Res* 14:424-32, 1996.

# CHAPTER 5

# IL-1 INDUCES TENSILE WEAKENING AND COLLAGEN DEGRADATION IN ARTICULAR CARTILAGE EXPLANTS

## 5.1 Abstract

Objective. To determine whether IL-1-induced tensile weakening is caused by the loss of glycosaminoglycan or the additional fragmentation of the collagen network.

Methods. Cartilage explants from superficial (including the articular surface), middle, and deep zones of cartilage were cultured for 1 or 3 weeks in basal medium with or without IL-1. The explants and culture medium were subsequently analyzed for the amount of glycosaminoglycan and cleaved, denatured, and total collagen. The explants were analyzed for tensile properties, including tensile ramp modulus, strength, and failure strain.

Results. The effect of IL-1 treatment on cartilage tensile properties and content was dependent on culture duration and depth of the explant from the articular surface. The tensile strength and failure strain of IL-1-treated samples were lower after 3, but not 1 week of culture. IL-1 induced a significant release of GAG into the medium and loss of GAG from the cartilage explants after 1 week of culture, but elicited no change

in total, cleaved, or denatured collagen in the medium or in the explants. After 3 weeks of culture, there was a significant release of GAG into the medium and loss of GAG from the cartilage explants, as well as an increase in the amount of collagen fragmentation.

Conclusions. IL-1 treatment induces tensile weakening in cartilage explants which is due, not exclusively to the loss of GAG from the cartilage explant, but also to the degradation of the collagen network, especially the cleavage of collagen molecules.

## 5.2 Introduction

The tensile integrity of cartilage decreases with age and with the onset of arthritic disease. In macroscopically normal adult human articular cartilage from the femoral condyles, the tensile strength and stiffness of the superficial layer decrease after reaching peak values at 24 yrs of age [22]. Tensile equilibrium moduli are lower in macroscopically normal cartilage adjacent to fibrillated and osteoarthritic cartilage, compared to young normal cartilage of the human knee joint [1]. This decrease in tensile integrity of cartilage could be attributed to an alteration of the organization and integrity of the collagen network [22]. Such weakening may be a downstream result of cell death [32, 38, 39, 50, 51] and an inability of the remaining cells to maintain tissue homeostasis. It may also directly reflect mechanical wear of the articular surface [31, 51], with physical disruption of the collagen network of the articular surface contributing to mechanical weakening. Enzymatic degradation of extracellular matrix components may also play a role with fragmentation of collagen molecules and proteoglycans resulting in a weakened extracellular matrix [2, 25]. However, the sequence of events leading to the tensile weakening of articular cartilage with age and degeneration remain unknown.

Cartilage tensile properties are dependent on the integrity of the collagen network. Enzymatic degradation of the collagen network with elastase, which eliminates covalent crosslinks of cartilage collagen [49], or collagenase, which cleaves the triple helical region of collagen, results in a ~45% or ~90% decrease in the tensile strength, respectively, of the superficial layer of macroscopically normal human

articular knee cartilage [2, 25]. In degenerate and osteoarthritic human articular cartilage from the femoral condyle, there is an increase in the percentage of degraded collagen [3]. In particular, increased cleaved [6] and denatured [19] collagen are evident in degenerate and osteoarthritic cartilage and are detectable by immunoassays as well as visible by immunohistochemistry [12, 13, 20]. It is a damaged collagen network in osteoarthritic cartilage which is thought to result in increased swelling [3, 27, 29, 30], increased instantaneous deformation [4], and an inability of the collagen network to withstand tensile stress [5]. Mechanical or enzymatic collagen degradation may be related to the overall loss of tensile integrity of OA cartilage and may underlie the sequence of events resulting in progressive degeneration and, ultimately, end-stage OA.

IL-1 is a mediator of inflammatory reactions and is thought to be a mediator of articular cartilage destruction in joint diseases. IL-1 is a cytokine produced by activated synoviocytes, mononuclear cells, and chondrocytes [36, 48, 52, 53], and elevated levels of IL-1 have been found in the synovial membrane, synovial fluid, and cartilage from patients suffering from osteoarthritis and, especially, rheumatoid arthritis [14, 46, 48, 53, 54, 59]. In explant culture, IL-1 induces both aggrecan cleavage and loss [37, 43], as well as collagen denaturation in articular cartilage of various species [7, 13, 26, 34]. IL-1 is thought to mediate these actions through the stimulation of a variety of cells, including synovial cells, fibroblasts, and chondrocytes, to synthesize and secrete metalloproteases [10, 21, 33]. Matrix metalloproteinases such as collagenase and stromelysin can degrade proteoglycan subunits [35] and type II collagen [60].

Collagen degradation in an IL-1 culture system may release multiple collagen components. The analysis of collagen in the medium and tissue would provide information on the collagen content and network structure (Figure 5.1). Intact collagen molecules may be synthesized by the cells and released into culture medium or remain in the residual tissue after alpha-chymotrypsin extraction. Cleaved and denatured collagen molecules may be released into the media or extracted from the tissue by alpha-chymotrypsin. Smaller soluble fragments of collagen molecules may also be present in the culture medium. These forms of collagen molecules were measured in this study as hydroxyproline in the medium, alpha-chymotrypsin extract and proteinase K digest. In the medium and alpha-chymotrypsin extract, cleaved and denatured collagens were measured by competitive ELISAs with the COL2-3/4C and COL2-3/4M antibodies, respectively.

In particular, there is a distinctive time-course of proteoglycan and collagen degradation induced by IL-1. Loss of proteoglycan in explant culture of articular cartilage with IL-1 occurs prior to the cleavage and denaturation of type II collagen [7, 11, 26]. This offers a unique culture system for the study of the effect of proteoglycan and collagen degradation on tensile properties of articular cartilage. Thus, it was hypothesized in this study that IL-1 induced matrix degradation causes tensile weakening and that this tensile weakening is related to specific alterations in the collagen network. Therefore, it was the objective of this study to determine if cytokine-induced tensile weakening of articular cartilage was due to the loss of glycosaminoglycan or the subsequent loss of collagen network integrity.

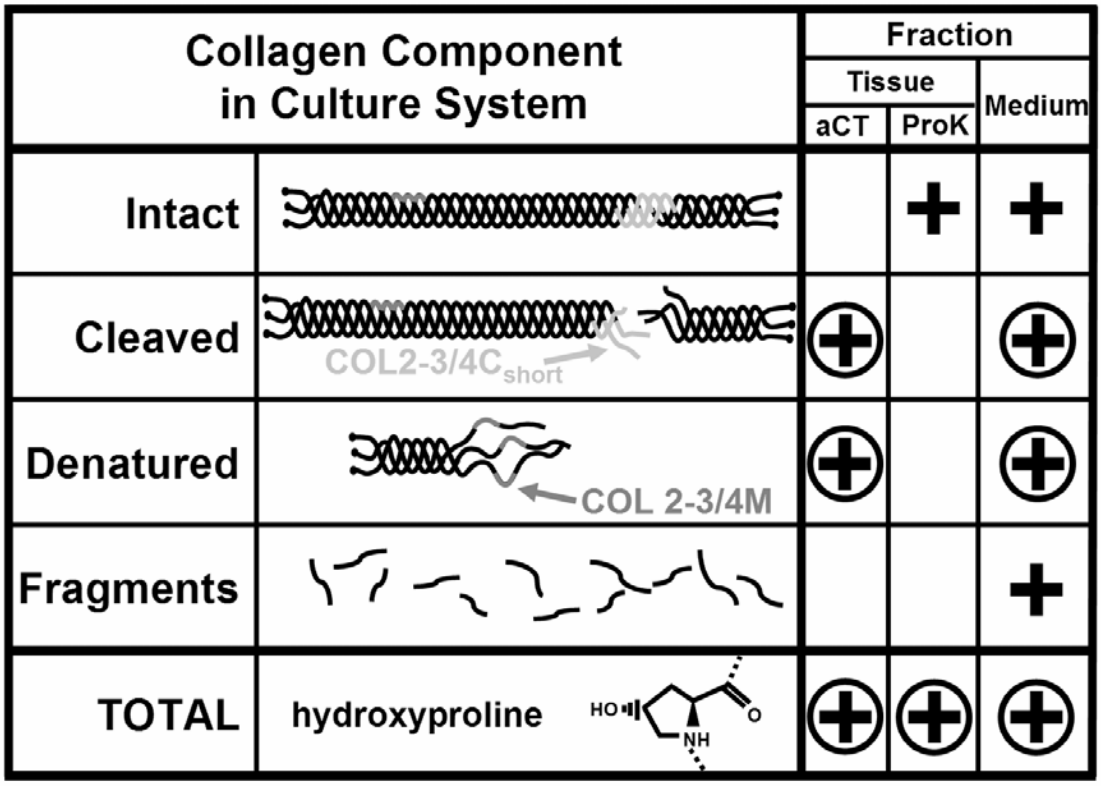



**Figure 5.1:** Measures of collagen network degradation after explant culture of cartilage. The components present (**+**) in tissue or medium fractions were intact collagen, cleaved collagen, denatured collagen, and smaller fragments of collagen. Amounts of cleaved collagen, denatured collagen, and total collagen were quantified (○) by analysis for COL2-3/4$C_{short}$ epitope, COL2-3/4M epitope, and hydroxyproline, respectively.

## 5.3 Materials and Methods

### 5.3.1 Materials

Materials for cartilage explant isolation and culture and the extraction of degraded collagen were obtained as described previously [3, 42]. RhIL-1α was obtained from R&D Systems Inc. (Minneapolis, MN). Enzyme-linked immunoabsorbent assays (ELISAs) to detect cleaved (using the polyclonal antibody, COL2-3/4C$_{short}$) and denatured (using the monoclonal antibody, COL2-3/4M) collagen epitopes were obtained from IBEX Diagnostics, Inc. (Montreal, Quebec, Canada).

### 5.3.2 Cartilage Isolation and Culture

Osteochondral blocks were obtained from medial and lateral aspects of the patellofemoral groove of young adult bovine knee joints (1-2 yrs old, n=4). From each block, cartilage slices, ~0.3 mm thick, were cut sequentially, parallel to the articular surface, to yield cartilage from the superficial (S), middle (M), and deep (D) regions. The S cartilage explants had an intact articular surface, and all explants were cut to an area of 10 mm length × 5 mm width. The cartilage explants were weighed wet, and then incubated in medium (DMEM with 10 mM HEPES, 0.1 mM nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 25 μg/ml ascorbate) supplemented with 0.01% bovine serum albumin (BSA) alone (basal medium) or with an additional 5 ng/ml rhIL-1α (IL-1). Cartilage explants were incubated at 37°C and 5% $CO_2$ with three medium changes weekly for 7 or 21 days. The spent medium from each sample was pooled together each week. After culture, each cartilage explant was weighed wet,

measured for thickness, and cut into a tapered specimen for mechanical testing and adjacent tissue. Adjacent tissue was used for biochemical analysis.

### 5.3.3 Biomechanical Analysis

Tapered tensile specimens were tested in tension and data were analyzed for the tensile ramp modulus, strength, and strain at failure. Each tapered tensile specimen had a gage area of 4 mm x 0.8 mm (L x W) and were elongated at a constant rate of extension (5 mm/min) until failure, while monitoring load. From the displacement and load data, tensile mechanical properties were determined. Stress, in units of megapascals (MPa), was calculated as load normalized to the width and thickness of the gage region. Strain (unitless) was calculated by normalizing the displacement data to the sample length. The ramp modulus was calculated as the slope of the stress-strain curve between 25% and 75% of the maximum strain, while the strength and failure strain were taken to be the point at which the maximum stress was achieved.

### 5.3.4 Biochemical Analysis

The surrounding residual tissue was analyzed for the quantity of matrix components, including cleaved and denatured collagen. The tissue was weighed wet and treated with 1 mg/ml alpha-chymotrypsin (aCT) for 18 hrs at 37°C to extract denatured collagen [3]. The residual tissue was solubilized with 0.5 mg/ml proteinase K (ProK) for 24 hrs at 60°C. The spent medium, aCT extracts, and ProK digests were analyzed for sulfated glycosaminoglycan (GAG) [15] and hydroxyproline [56]. Because the amount of hydroxyproline in medium was small, medium from multiple blocks from the same animal were pooled together and analyzed. The spent medium and aCT extracts were analyzed for cleaved (COL2-3/4$C_{short}$) and denatured (COL2-

3/4M) collagen by ELISA. Hydroxyproline content was converted to collagen (COL) content using a mass ratio of collagen to hydroxyproline equal to 7.25 [18]. Sulfated GAG content was calculated by comparison to known concentrations of shark chondroitin sulfate. The contents of GAG, COL, COL2-3/4$C_{short}$ and COL2-3/4M were calculated as the mass or molar mass normalized to wet weight. The percent of COL in aCT (degraded) was calculated as that in aCT compared to the sum in aCT and ProK solutions.

### 5.3.5 Statistical Analysis

Data are expressed as mean ± SEM. The effect of IL-1 on mechanical and biochemical parameters was assessed using repeated measures ANOVA with tissue depth (S, M, D) as a repeated factor, with 1-week and 3-week experiments being performed, and analyzed, separately. For analysis of medium, week in culture ($1^{st}$, $2^{nd}$, or $3^{rd}$ week) was an additional repeated factor. When IL-1 or depth from the articular surface had an effect (for $p<0.05$), planned comparisons were made between treatment groups at each depth.

## 5.4 Results

**Biomechanical Analysis.** IL-1 treatment lowered the tensile integrity of cartilage samples in a manner dependent on the culture duration and layer (depth from the articular surface). In particular, the tensile integrity of IL-1-treated samples, compared to those incubated in basal medium, was lower after 3 weeks, but not 1 week of culture. After 1 week of culture, the tensile ramp modulus (Figure 5.2A), strength

(Figure 5.2C), and failure strain (Figure 5.2E) of samples cultured in medium with IL-1 were similar to those cultured in basal medium ($p=0.3$, $p=0.5$, $p=0.3$, respectively). Each of these tensile properties was dependent on layer (each $p<0.005$), without an interaction effect between layer and IL-1 treatment ($p=0.5$-$0.8$). Planned comparisons of treatment at each depth revealed no significant effect of IL-1 treatment after only 1 week of culture for ramp modulus ($p=0.2$-$0.5$), strength ($p=0.2$-$0.8$), or failure strain ($p=0.4$-$0.9$).

In contrast, after 3 weeks of culture, treatment with IL-1 caused a decrease in tensile strength and failure strain of cartilage explants, with effects most pronounced in S and M samples. After 3 weeks of culture, IL-1 treated samples showed a tendency for an overall decrease in tensile strength (Figure 5.2D), with a decrease in S (31%, $p<0.05$) samples, a tendency for a decrease in M (25%, $p=0.18$) samples, and no effect on D (5%, $p=0.7$) samples. Failure strain (Figure 5.2F) was also affected by IL-1 treatment, being lower in IL-1 treated S (33%, $p<0.05$) and M (38%, $p<0.005$) samples but not D (16%, $p=0.2$) samples. Ramp modulus (Figure 5.2B) was not affected by IL-1 treatment in S, M, or D samples ($p=0.2$-$0.8$).

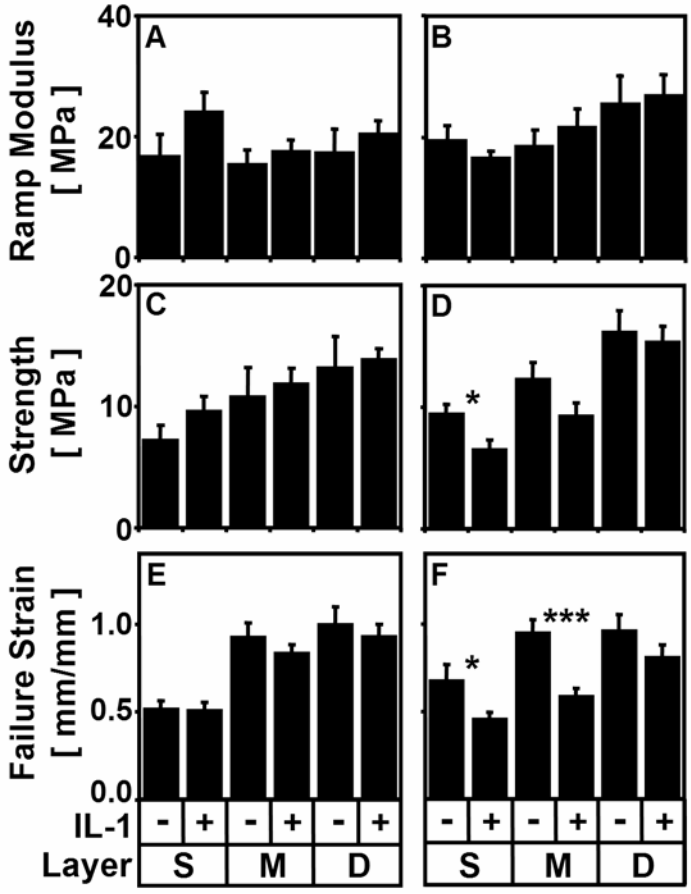



**Figure 5.2:** Effect of IL-1 on cartilage explant tensile properties. Superficial (S), middle (M), and deep (D) layer cartilage explants were incubated with (**+**) or without (**–**) 5 ng/ml IL-1α for 1 (**A**, **C**, **E**) or 3 (**B**, **D**, **F**) weeks and analyzed for (**A**, **B**) tensile ramp modulus, (**C**, **D**) strength, and (**E**, **F**) strain at failure. n=7-16, *p<0.05, ***p<0.005.

**Biochemical Analysis.** IL-1 treatment had a significant effect on cartilage explants, in terms of the release and retention of matrix components. The effects were dependent on the culture duration and the layer of the explant. Release of matrix components into the medium was stimulated by IL-1 treatment, with the release of collagen network components being delayed relative to the release of GAG. Because the pattern of release of matrix components into the medium from 1-week samples was similar to that released from 3-week samples after 1 week of culture, data is only shown for the 3-week samples.

In particular, IL-1 stimulated the release of GAG from cartilage samples by 1 week of culture ($p<0.005$), with an effect on cumulative release after 2 and 3 weeks of culture (Figure 5.3A); this release was dependent on culture duration ($p<0.005$) and layer ($p<0.05$) with an interactive effect of duration and layer ($p<0.001$). After 1 week of culture, GAG released into the medium was significantly higher in IL-1 treated S (116%, $p<0.005$), M (91%, $p<0.005$), and D (95%, $p<0.005$) samples (Figure 5.3A). The effect of IL-1 on cumulative GAG release was lower after 2 and 3 weeks, but cumulative GAG release from IL-1-treated S, M, and D samples remained high after 2 weeks of culture (45-70%, each $p<0.05$) and tended to be higher after 3 weeks of culture (20-45%, $p=0.082$, $p<0.05$, and $p=0.14$, respectively) compared to samples cultured in basal medium.

The effect of IL-1 on the release of COL (determined from hydroxyproline) into the medium was delayed relative to the IL-1-enhanced release of GAG into the medium. IL-1 induced release of COL from cartilage samples ($p<0.05$, Figure 5.3B), with the release being dependent on culture duration ($p<0.005$) but not layer ($p=0.67$)

and without an interaction effect (p=0.78). COL release from IL-1 treated samples during week 1 of culture was slightly higher (105-177%) than that from samples cultured in basal medium. However, IL-1 increased the release of COL dramatically during subsequent weeks of culture, being higher by 156-368% at week 2 and higher by 213-788% at week 3.

The effect of IL-1 on the release of the collagen degradation markers, COL2-3/4$C_{short}$ and COL2-3/4M epitopes, into the medium was also delayed relative to the IL-1-enhanced release of GAG into the medium. IL-1 enhanced the release of COL2-3/4$C_{short}$ (Figure 5.3C) and COL2-3/4M (Figure 5.3D) epitopes (each $p<0.01$). The cumulative release of COL2-3/4$C_{short}$ was dependent on culture duration ($p<0.005$) and layer ($p<0.005$), with an interaction effect ($p<0.005$). After 1 week of culture, the release of COL2-3/4$C_{short}$ epitope was not enhanced by IL-1 treatment in S, M, or D samples (p=0.17-0.47). COL2-3/4$C_{short}$ release during week 2 was higher in IL-1-stimulated S samples (125%, $p<0.05$), had a tendency to be higher in M samples (155%, p=0.052), and had little effect in D samples (71%, p=0.3). During week 3, the release was higher in IL-1-treated S (548%, $p<0.005$) and M (373%, $p<0.005$) samples but not D (178%, p=0.15) samples.

The cumulative release of COL2-3/4M was also dependent on culture duration ($p<0.005$) but not layer (p=0.30), and with a slight interaction effect (p=0.07). After 1 week of culture, COL2-3/4M epitope was not enhanced by IL-1 treatment in S, M, or D samples (p=0.11-0.41). In contrast, IL-1 did enhance the cumulative release of the COL2-3/4M epitope during week 2 of culture, being higher in IL-1-treated S (596%, $p<0.005$) and M (517%, $p<0.05$) samples and having a tendency to be higher in D

(285%, $p=0.13$) samples. During week 3, the release was higher in IL-1-stimulated S (2503%, $p<0.005$) and M (1350%, $p<0.05$) samples with a tendency of being higher in D (958%, $p=0.082$) samples.

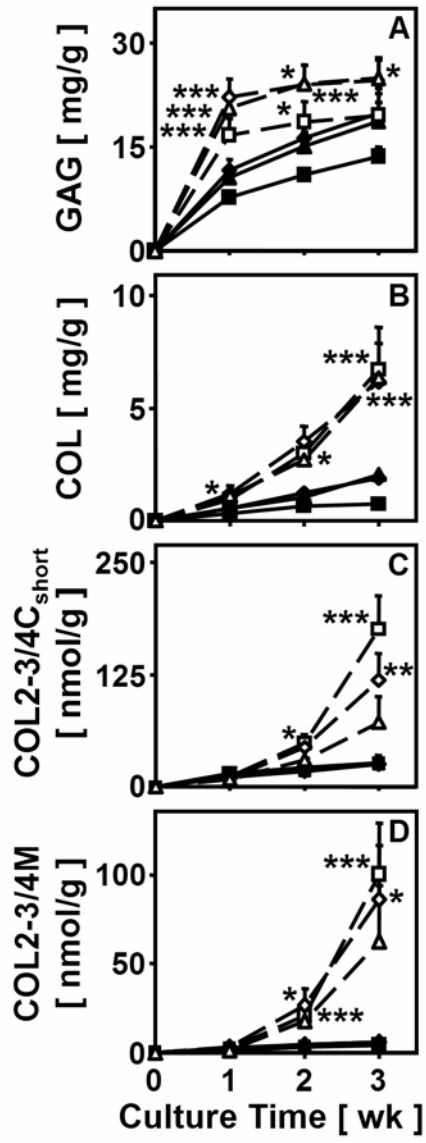



**Figure 5.3:** Effect of IL-1 on the cumulative release of matrix components into medium. Superficial (□, ■), middle (Δ, ▲), and deep (○, ●) cartilage explants were cultured for 3 weeks with (▬ ▬, □, Δ, ○) or without (——, ■, ▲, ●) 5 ng/ml IL-1α and analyzed for the cumulative release into medium of (**A**) glycosaminoglycan (GAG), (**B**) collagen (COL), (**C**) cleaved collagen (COL2-3/4$C_{short}$), and (**D**) denatured collagen (COL2-3/4M). n=14-16, *p<0.05, **p<0.01, ***p<0.005.

Consistent with the release of matrix components into the medium, the degradative effect of IL-1 on the residual collagen network in the cartilage samples was delayed relative to the decrease of residual GAG. After 1 and 3 weeks of culture, IL-1 decreased the amount of GAG (each $p<0.005$, Figure 5.4A, B) remaining in the cartilage samples, with the amounts being dependent on layer after the 3-week ($p<0.005$) but not the 1-week ($p=0.2$) culture duration. After 1 week of culture, the amount of residual GAG in IL-1-treated samples (Figure 5.4A) was 53-75% lower in S, M, and D samples (each $p<0.05$) compared to samples cultured in basal medium. The amount of GAG remaining in the IL-1-treated samples after 3 weeks of culture (Figure 5.4B) was considerably lower in S (79%, $p<0.005$), M (81%, $p<0.005$), and D (74%, $p<0.005$) samples. Approximately 94% of the total GAG from BSA samples and 85% from IL-1 samples were aCT-extractable.

In contrast, IL-1 had no effect on the amount of COL remaining in cartilage samples after 1 or 3 weeks of culture. COL remaining in the cartilage samples after 1 and 3 weeks of culture was similar between samples cultured in basal medium and those cultured in medium with IL-1 ($p=0.9$, $p=0.3$, respectively, Figure 5.4C, D).

While it had no effect on the amount of COL remaining in the cartilage samples, IL-1 increased the percentage of COL in the aCT fraction in a manner that was delayed relative to the time course of decrease in GAG but that paralleled the time course for decrease in tensile integrity. The percentage of aCT-extractable COL was significantly higher after 3 weeks ($p<0.005$, Figure 5.4F) but not after 1 week ($p=0.1$, Figure 5.4E) of IL-1 treatment. This was dependent on layer for 1-week and 3-week samples ($p<0.005$, $p<0.01$, respectively). The percent of COL in aCT was higher after

the 3-week culture duration in S (102%, $p<0.005$) and M (39%, $p<0.005$) but not D ($p=0.3$) samples (Figure 5.4F). The percent of COL in aCT was not higher after the 1-week culture duration in S, M, or D samples ($p=0.6$-$0.9$, Figure 5.4E).

IL-1 treatment had a differential effect on the amount of COL2-3/4$C_{short}$ and COL2-3/4M epitopes found in the aCT extracts, with the delay of IL-1 induced collagen network degradation being evident between 1 and 3 week samples. The presence of the collagen cleavage marker, COL2-3/4$C_{short}$, tended to be higher in samples cultured in IL-1 for 3 weeks ($p=0.055$, Figure 5.4H) and tended to depend on layer ($p=0.08$) but was not higher in samples cultured in IL-1 for only 1 week ($p=0.2$, Figure 5.4G). After the 3-week culture duration, the amount of COL2-3/4$C_{short}$ was significantly higher in IL-1-treated S (95%, $p<0.05$) but not in M or D ($p=0.1$-$0.6$) samples. The presence of the COL2-3/4M epitope, though dependent on layer (each $p<0.05$), was not increased by IL-1 treatment after the 1-week ($p=0.9$, Figure 5.4I) or 3-week ($p=0.6$, Figure 5.4J) culture duration.

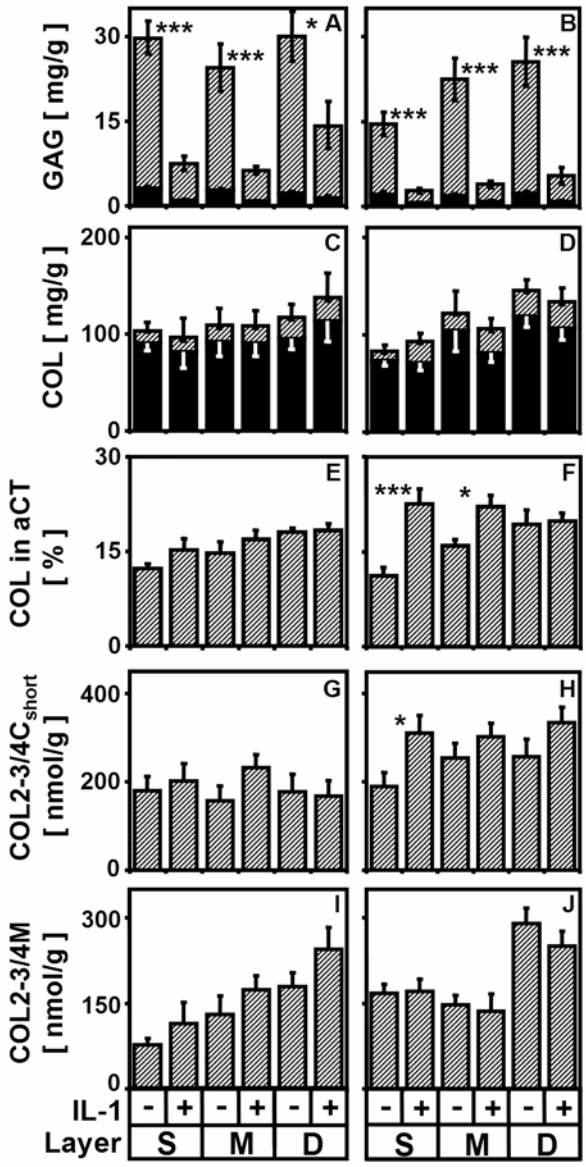



**Figure 5.4:** Effect of IL-1 on the amount of matrix components remaining in cartilage explants. Superficial (S), middle (M), and deep (D) layer cartilage explants were cultured for 1 (**A**, **C**, **E**, **G, I**) or 3 (**B**, **D**, **F**, **H, J**) weeks with (**+**) or without (**–**) 5 ng/ml IL-1α and analyzed for (**A**, **B**) GAG, (**C**, **D**) COL, (**E**, **F**) COL in aCT, (**G**, **H**) COL2-3/4$C_{short}$, and (**I**, **J**) COL2-3/4M. Degraded collagen was first extracted with alpha-chymotrypsin (▨) and residual tissue was digested with proteinase K (■). n=7-16, *p<0.05, ***p<0.005

## 5.5 Discussion

IL-1 induced tensile weakening and stimulated matrix degradation, including loss of GAG and degradation of COL, in adult bovine cartilage explants. Because of the unique time course of IL-1 induced matrix degradation, it was shown that the IL-1-induced loss of GAG did not, by itself, cause a loss of tensile integrity but that the loss of tensile strength may be related to specific alterations in the collagen network. IL-1 initially (within the first week of culture) reduced the amount of GAG remaining in the matrix (Figure 5.4A), without an associated decrease in tensile strength or failure strain (Figure 5.2C, E) or effect on the collagen network, including the total amount of COL remaining in the cartilage samples (Figure 5.4C), the percentage of degraded collagen (COL in aCT, Figure 5.4E), the amount of cleaved collagen (COL2-3/4C, Figure 5.4G) or the amount of denatured collagen (COL2-3/4M, Figure 5.4I). In contrast, after 3 weeks of culture, while the amount of GAG remaining in the cartilage samples was still low, there was an IL-1 associated decrease in tensile strength (Figure 5.2D) and failure strain (Figure 5.2F), as well as an increase in the percentage of degraded collagen (Figure 5.4F) and cleaved collagen (Figure 5.4H). The decrease in tensile strength seen in this study may be directly related to the alterations of the collagen network, and these may be similar to the tensile weakening and collagen degradation seen in osteoarthritis [1, 6, 19].

Certain factors may limit the interpretation of the results of this study and the relationships between alterations of the extracellular matrix and the mechanical integrity of articular cartilage. One factor to consider is the use of adult bovine

articular cartilage in a model of osteoarthritis. Adult, skeletally mature animals were chosen, as opposed to animals still in stages of development and growth [55], adult cartilage being more appropriate for models of disease such as in this study. Further, the adult bovine knee joint offers a broad, relatively flat surface from which to obtain cartilage tensile samples, and cartilage can be obtained from a skeletally mature animal that is generally free of cartilage degeneration. However, certain compositional, structural, and functional differences exist between human and bovine articular cartilage. Adult bovine articular cartilage has lower tensile stiffness and strength [8, 41, 47, 55, 57, 58] than that of adult human cartilage [1, 22, 40, 51]. The depth-dependent variation in tensile strength and stiffness of adult bovine articular cartilage [8] is unlike that of adult human cartilage [22, 51]. However, the failure strain [8, 41] of adult bovine cartilage is consistent in magnitude and depth-variation with that of young adult human cartilage [51]. Generally, collagen content is slightly lower and GAG content slightly higher in bovine [55] than in human [24, 28, 51] articular cartilage, and the crosslink composition of the collagen network can vary, with hydroxypyridinoline crosslinks being less abundant in the adult bovine [55] than in adult human cartilage [4]. Despite the few differences in composition, structure, and function between bovine and human adult articular cartilage, there was ample support for the use of adult bovine articular cartilage, and samples that were consistent, reproducible, and without signs of cartilage fibrillation or erosion were obtained.

Use of IL-1 in tissue culture as a model of osteoarthritis also requires certain considerations. Addition of IL-1 to the culture system is known to result in cartilage degradation with loss of matrix components similar to that in OA. However, in such a

culture system, cartilage matrix degradation occurs in the absence of mechanical loading and surrounding joint fluids and tissues. However, IL-1, previously identified as catabolin, is abnormally elevated in diseases such as OA and RA, and it is often used in culture systems to identify cascades of enzymatic activity associated with osteoarthritis such as that of aggrecanase and matrix metalloproteinases [6, 9, 16, 26, 44, 45]. Using IL-1 in the culture of cartilage tissue explants in the present study allowed study of the effects of experimentally-induced matrix degradation alone on mechanical integrity.

The tensile properties measured in this study were consistent with previous studies of adult bovine articular cartilage from the patellofemoral groove [8, 55] and extend previous knowledge of the relationship between the tensile integrity of cartilage and the condition of the collagen network. While it is unknown precisely what amounts of collagen crosslinks were degraded or the amount of collagen that was cleaved or denatured in previous studies, they showed a dramatic decrease (45-90%) in the tensile strength of the superficial layer of human articular cartilage following enzymatic degradation of the collagen network [2, 25]. This study showed a 31% decrease in tensile strength of the superficial layer of bovine articular cartilage following culture with IL-1. This may reflect a lower level of active matrix metalloproteinases (MMPs) induced by IL-1 compared to levels of active MMPs or enzymes used in previous studies, or it may reflect species-associated differences. Culture with IL-1 induced tensile weakening in articular cartilage explants and highlighted the role of collagen degradation.

The relative amounts of cleaved and denatured collagen, as well as GAG, released into media and remaining in residual cartilage samples following culture with IL-1 were similar to those determined previously [7]. Further, the time course of delayed collagen network degradation with respect to the release and loss of GAG from cartilage samples [7, 26] has been reported previously. IL-1 seemed to exert its greatest effect on the collagen network of S and M samples, agreeing with previous studies in which chondrocytes isolated from the superficial zone of carilage and cultured in agarose were more sensitive to IL-1 than those of the deep zone [17]. The present study expands upon previous studies by highlighting the cumulative effect of GAG loss and collagen degradation in cartilage samples, as opposed to GAG loss alone, as a factor in cartilage tensile weakening. In fact, IL-1 induced a two-fold increase in degraded collagen, a difference similar to that between osteoarthritic and non-arthritic cartilage [6, 19], but induced only a 30% decrease in tensile strength, about half the difference seen between normal and fibrillated cartilage [23]. This indicates that there may be additional or other forms of enzymatic or mechanical cartilage degradation which contribute to the tensile weakening observed in osteoarthritis.

Further study is needed to determine what initiates the tensile weakening observed in arthritic disease and causes the progression of disease. This study showed that the culture of cartilage explants with IL-1 did have an effect on the cartilage tensile properties, and that the tensile weakening following IL-1 treatment was due, not exclusively to the loss of GAG, but to degradation of the collagen network, specifically cleavage of collagen molecules. It is the strength of the collagen network

which is thought to constrain the proteoglycans within cartilage and prevent the swelling observed in osteoarthritis. While IL-1 might not be the only contributor to the pathogenesis of osteoarthritis, it may be an initiator or propagator of osteoarthritic cartilage destruction.

## 5.6 Acknowledgments

This work was supported by the Arthritis Foundation, the National Institutes of Health, and the National Science Foundation. The dissertation author (primary investigator) thanks the coauthors of the manuscript for their contributions: Yang Xue and Michael Chen.

## 5.7 References

1. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH: Tensile properties of human knee joint cartilage: I. influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus. *J Orthop Res* 4:379-92, 1986.

2. Bader DL, Kempson GE, Barrett AJ, Webb W: The effects of leucocyte elastase on the mechanical properties of adult human articular cartilage in tension. *Biochim Biophys Acta* 677:103-8, 1981.

3. Bank RA, Krikken M, Beekman B, Stoop R, Maroudas A, Lafeber FPJG, Te Koppele JM: A simplified measurement of degraded collagen in tissues: application in healthy, fibrillated and osteoarthritic cartilage. *Matrix Biol* 16:233-43, 1997.

4. Bank RA, Soudry M, Maroudas A, Mizrahi J, TeKoppele JM: The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. *Arthritis Rheum* 43:2202-10, 2000.

5. Basser PJ, Schneiderman R, Bank RA, Wachtel E, Maroudas A: Mechanical properties of the collagen network in human articular cartilage as measured by osmotic stress technique. *Arch Biochem Biophys* 351:207-19, 1998.

6. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chan J, Van Wart H, Poole AR: Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* 99:1534-45, 1997.

7. Billinghurst RC, Wu W, Ionescu M, Reiner A, Dahlberg L, Chen J, van Wart H, Poole AR: Comparison of the degradation of type II collagen and proteoglycan in nasal and articular cartilages induced by interleukin-1 and the selective inhibition of type II collagen cleavage by collagenase. *Arthritis Rheum* 43:664-72, 2000.

8. Chen AC, Temple MM, Ng DM, DeGroot J, Verzijl N, TeKoppele JM, Sah RL: Induction of advanced glycation endproducts alters tensile properties of articular cartilage. *Arthritis Rheum* 46:3212-7, 2002.

9. Chubinskaya S, Huch K, Mikecz K, Cs-Szabo G, Hasty KA, Kuettner KE, Cole AA: Chondrocyte matrix metalloproteinase-8: up-regulation of neutrophil collagenase by interleukin-1 beta in human cartilage from knee and ankle joints. *Lab Invest* 74:232-40, 1996.

10. Dayer JM, de Rochemonteix B, Burrus B, Demczuk S, Dinarello CA: Human recombinant interleukin 1 stimulates collagenase and prostaglandin E2 production by human synovial cells. *J Clin Invest* 77:645-8, 1986.

11. Dingle JT, Horsfield P, Fell HB, Barratt ME: Breakdown of proteoglycan and collagen induced in pig articular cartilage in organ culture. *Ann Rheum Dis* 34:303-11, 1975.

12. Dodge GR, Pidoux I, Poole AR: The degradation of type II collagen in rheumatoid arthritis: an immunoelectron microscopic study. *Matrix* 11:330-8, 1991.

13. Dodge GR, Poole AR: Immunohistochemical detection and immunochemical analysis of type II collagen degradation in human normal rheumatoid, and osteoarthritis articular cartilage and in explants of bovine articular cartilage cultured with interleukin 1. *J Clin Invest* 83:647-61, 1989.

14. Farahat MN, Yanni G, Poston R, Panayi GS: Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. *Ann Rheum Dis* 52:870-5, 1993.

15. Farndale RW, Buttle DJ, Barrett AJ: Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 883:173-7, 1986.

16. Flannery CR, Hughes CE, Schumacher BL, Tudor D, Aydelotte MB, Kuettner KE, Caterson B: Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and Is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism. *Biochem Biophys Res Commun* 254:535-41, 1999.

17. Häuselmann HJ, Flechtenmacher J, Michal L, Thonar EJ-MA, Shinmei M, Kuettner KE, Aydelotte MB: The superficial layer of human articular cartilage is more susceptible to interleukin-1-induced damage than the deeper layers. *Arthritis Rheum* 39:478-88, 1996.

18. Herbage D, Bouillet J, Bernengo J-C: Biochemical and physicochemical characterization of pepsin-solubilized type-II collagen from bovine articular cartilage. *Biochem J* 161:303-12, 1977.

19. Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, Poole AR: Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. *J Clin Invest* 93:1722-32, 1994.

20. Hollander AP, Pidoux I, Reiner A, Rorabeck C, Bourne R, Poole AR: Damage to type II collagen in aging and osteoarthritis starts at the articular surface, orginates around chondrocytes, and extends into the cartilage with progressive degeneration. *J Clin Invest* 96:2859-69, 1995.

21. Jasin HE, Dingle JT: Human mononuclear cell factors mediate cartilage matrix degradation through chondrocyte activation. *J Clin Invest* 68:571-81, 1981.

22. Kempson GE: Relationship between the tensile properties of articular cartilage from the human knee and age. *Ann Rheum Dis* 41:508-11, 1982.

23. Kempson GE, Freeman MAR, Swanson SAV: Tensile properties of articular cartilage. *Nature* 220:1127-8, 1968.

24. Kempson GE, Muir H, Pollard C, Tuke M: The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. *Biochim Biophys Acta* 297:456-72, 1973.

25. Kempson GE, Tuke MA, Dingle JT, Barrett AJ, Horsfield PH: The effects of proteolytic enzymes on the mechanical properties of adult human articular cartilage. *Biochim Biophys Acta* 428:741-60, 1976.

26. Kozaci LD, Buttle DJ, Hollander AP: Degradation of type II collagen, but not proteoglycan, correlates with matrix metalloproteinase activity in cartilage explant cultures. *Arthritis Rheum* 40:164-74, 1997.

27. Maroudas A: Balance between swelling pressure and collagen tension in normal and degenerate cartilage. *Nature* 260:808-9, 1976.

28. Maroudas A, Bayliss MT, Venn MF: Further studies on the composition of human femoral head cartilage. *Ann Rheum Dis* 39:514-23, 1980.

29. Maroudas A, Venn M: Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. II. Swelling. *Ann Rheum Dis* 36:399-406, 1977.

30. Maroudas A, Ziv I, Weisman N, Venn M: Studies of hydration and swelling pressure in normal and osteoarthritic cartilage. *Biorheology* 22:159-69, 1985.

31. Meachim G: Light microscopy of Indian ink preparations of fibrillated cartilage. *Ann Rheum Dis* 31:457-64, 1972.

32. Mitrovic D, Quintero M, Stankovic A, Ryckewaert A: Cell density of adult human femoral condylar articular cartilage. *Lab Invest* 49:309-16, 1983.

33. Mizel SB, Dukovich M, Rothstein J: Preparation of goat antibodies against interleukin 1: use of an immunoadsorbent to purify interleukin 1. *J Immunol* 131:1834-7, 1983.

34. Mort JS, Dodge GR, Roughley PJ, Liu J, Finch SJ, Dipasquale G, Poole AR: Direct evidence for active metalloproteinases mediating matrix degradation in interleukin 1-stimulated human articular cartilage. *Matrix* 13:95-102, 1993.

35. Nguyen Q, Murphy G, Roughley PJ, Mort JS: Degradation of proteoglycan aggregate by a cartilage metalloproteinase. Evidence for the involvement of stromelysin in the generation of link protein heterogeneity in situ. *Biochem J* 259:61-7, 1989.

36. Ollivierre F, Gubler U, Towle CA, Laurencin C, Treadwell BV: Expression of IL-1 genes in human and bovine chondrocytes: a mechanism for autocrine control of cartilage matrix degradation. *Biochem Biophys Res Commun* 141:904-11, 1986.

37. Poole AR: Imbalances of anabolism and catabolism of cartilage matrix components in osteoarthritis. In: *Osteoarthritic Disorders,* ed. by KE Kuettner, Goldberg VM, American Academy of Orthopaedic Surgeons, Rosemont, IL, 1995, 247-60.

38. Quintero M, Mitrovic DR, Stankovic A, de Seze S, Miravet L, Ryckewaert A: Aspects cellulaires du vieillissement du cartilage articulaire. I. cartilage condylien a surface normale, preleve dans les genoux normaux. *Revue du Rhumatisme* 51:375-9, 1984.

39. Quintero M, Mitrovic DR, Stankovic A, de Seze S, Miravet L, Ryckewaert A: Aspects cellulaires du vieillissement du cartilage articulaire. II. cartilage condylien a surface fissuree preleve dans les genoux normaux et arthrosiques. *Revue du Rhumatisme* 51:445-9, 1984.

40. Roberts S, Weightman B, Urban J, Chappell D: Mechanical and biochemical properties of human articular cartilage from the femoral head after subcapital fracture. *J Bone Joint Surg Br* 68-B:418-22, 1986.

41. Roth V, Mow VC: The intrinsic tensile behavior of the matrix of bovine articular cartilage and its variation with age. *J Bone Joint Surg Am* 62-A:1102-17, 1980.

42. Sah RL, Kim YJ, Doong JH, Grodzinsky AJ, Plaas AHK, Sandy JD: Biosynthetic response of cartilage explants to dynamic compression. *J Orthop Res* 7:619-36, 1989.

43. Saklatvala J, Sarsfield SJ, Pilsworth LM: Characterization of proteins from human synovium and mononuclear leucocytes that induce resorption of cartilage proteoglycan in vitro. *Biochem J* 209:337-44, 1983.

44. Sandy JD, Gamett D, Thompson V, Verscharen C: Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 or retinoic acid can be inhibited by glucosamine. *Biochem J* 335:59-66, 1998.

45. Sandy JD, Neame PJ, Boynton RE, Flannery CR: Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. *J Biol Chem* 266:8683-5, 1991.

46. Schlaak JF, Pfers I, Meyer Zum Buschenfelde KH, Marker-Hermann E: Different cytokine profiles in the synovial fluid of patients with osteoarthritis, rheumatoid arthritis and seronegative spondylarthropathies. *Clin Exp Rheumatol* 14:155-62, 1996.

47. Schmidt MB, Mow VC, Chun LE, Eyre DR: Effects of proteoglycan extraction on the tensile behavior of articular cartilage. *J Orthop Res* 8:353-63, 1990.

48. Smith MD, Triantafillou S, Parker A, Youssef PP, Coleman M: Synovial membrane inflammation and cytokine production in patients with early osteoarthritis. *J Rheumatol* 24:365-71, 1997.

49. Starkey PM: The effect of human neutrophil elastase and cathepsin G on the collagen of cartilage, tendon, and cornea. *Acta Biol Med Ger* 36:1549-54, 1977.

50. Stockwell RA: The cell density of human articular and costal cartilage. *J Anat* 101:753-63, 1967.

51. Temple MM, Bae WC, Rivard KL, Sah RL: Age- and site-associated biomechanical weakening of human articular cartilage of the femoral condyle: relationship to cellularity and wear. *Trans Orthop Res Soc* 27:84, 2002.

52. Tetlow LC, Adlam DJ, Woolley DE: Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. *Arthritis Rheum* 44:585-94, 2001.

53. Towle CA, Hung HH, Bonassar LJ, Treadwell BV, Mangham DG: Detection of interleukin-1 in the cartilage of patients with osteoarthritis: a possible autocrine/paracrine role in pathogenesis. *Osteoarthritis Cartilage* 5:293-300, 1997.

54. Westacott CI, Whicher JT, Barnes IC, Thompson D, Swan AJ, Dieppe PA: Synovial fluid concentrations of five different cytokines in rheumatic diseases. *Ann Rheum Dis* 49:676-81, 1990.

55. Williamson AK, Chen AC, Masuda K, Thonar EJ-MA, Sah RL: Tensile mechanical properties of bovine articular cartilage: variations with growth and relationships to collagen network components. *J Orthop Res* 21:872-80, 2003.

56. Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 93:440-7, 1961.

57. Woo SL-Y, Akeson WH, Jemmott GF: Measurements of nonhomogeneous directional mechanical properties of articular cartilage in tension. *J Biomech* 9:785-91, 1976.

58. Woo SL-Y, Lubock P, Gomez MA, Jemmott GF, Kuei SC, Akeson WH: Large deformation nonhomogeneous and directional properties of articular cartilage in uniaxial tension. *J Biomech* 12:437-46, 1979.

59. Wood DD, Ihrie EJ, Dinarello CA, Cohen PL: Isolation of an interleukin-1-like factor from human joint effusions. *Arthritis Rheum* 26:975-83, 1983.

60. Wu J-J, Lark MW, Chun LE, Eyre DR: Sites of stromelysin cleavage in collagen types II, IX, X, and XI of cartilage. *J Biol Chem* 266:5625-8, 1991.

# CHAPTER 6

# CONCLUSIONS

## 6.1 Summary of Findings

The overall objective of this work was to further the understanding of the causal mechanisms of aging and osteoarthritis (OA) in cartilage as related to mechanical weakening, by assessing the sequence of events that occur in normal adult human articular cartilage with age and characteristics of cartilage that change with the progression to OA. The major findings of this work, related to this objective were:

1. Macroscopically normal (NL) adult human articular cartilage of the femoral condyle weakened with age, occurring earlier in the medial than the lateral femoral condyle (MFC, LFC, Chapter 2). These changes coincided with or preceded certain changes in surface structure, cell density and biochemical composition. Age-associated patterns of change are illustrated in Figure 2.4.
    a. The superficial layer of cartilage of the LFC was affected earliest with the tensile ramp modulus and strength being lower by ~21 and 37% in Middle and ~30 and 40% in Old than Young age groups. The middle and deep layers were mildly affected at a later age, if at all. The tensile strength and stiffness of MFC cartilage was low even at the Young age.

b. Wear of the articular surface coincided with changes in tensile properties. The mean reflectance score was high in LFC samples of all age groups and decreased from Young to Middle age in the MFC. The variance of the reflectance score, an index of surface roughness, increased in a manner parallel to the weakening of cartilage, with the variance being higher in the LFC Middle than Young age group and being high in all age groups of the MFC. Changes in the histopathology index paralleled changes in the reflectance score.

c. Changes in biochemical constituents occurred at a stage subsequent to changes in tensile properties or in zones which did not correspond to tensile weakening. DNA content decreased, not in the LFC, but in the superficial layer of the MFC from Young to Middle age groups. The intrinsic fluorescence increased with age group in all layers of the LFC, but not in a manner parallel to changes in tensile properties. Glycosaminoglycan (GAG) content was lower in Old than Middle age groups in the middle and deep, but not superficial, layers at the LFC and MFC sites.

2. Cartilage exhibiting mild surface fibrillation was weak compared to age-matched macroscopically normal cartilage (Chapter 3). Cartilage weakening in early cartilage degeneration paralleled changes in matrix content and collagen network remodeling (loss of intrinsic fluorescence). Changes in these properties in early cartilage degeneration are illustrated in Figure 3.4.

   a. The tensile properties were lower in mildly degenerate (DGN) and OA samples than NL samples at both the LFC and MFC sites and in all layers. Noteable were the ~85 and 78% lower equilibrium modulus and ~75 and 69%

lower strength in DGN and OA samples, respectively, than NL samples in the superficial layer of the LFC.

b. Indices of surface wear were high in DGN and OA samples of the LFC and in NL, DGN, and OA samples of the MFC, corresponding to mild fibrillation of DGN and OA samples and age-associated surface roughness of NL samples of the MFC.

c. Changes in biochemical constituents corresponding to changes in tensile properties were the decrease in the fluorescence ratio and an increase in collagen degradation. There was an increase in water content in OA samples which led to variable decreases in DNA and collagen when normalized to tissue wet weight.

3. Articular cartilage cell density showed mild decreases with age in macroscopically normal cartilage and noteable decreases in mildly degenerate articular cartilage of the MFC (Chapter 4). These changes were evidenced as alterations of cell organization in *en face* views of mildly degenerate cartilage.

   a. The cell density when determined by cell counting in vertical histological sections and by cell counting in *en face* views was lower in Aged DGN samples by ~43% in both cases when compared to Young NL samples. A mild age-associated decrease in cell density was evident in cell counts from *en face* views.

   b. The decrease in cell density was reflected in two dimensional projections of the top 25μm of cartilage with Young NL and Aged NL samples having

relatively large cell clusters and Aged DGN samples having relatively smaller clusters.

4. Matrix degradation of articular cartilage, induced by IL-1, altered the tensile strength and failure strain of articular cartilage (Chapter 5). The time course of matrix degradation by IL-1 was strongly suggestive of collagen degradation, rather than or coincident with GAG loss, as having a causal role in tensile weakening.
    a. Tensile weakening, observed as a decrease in tensile strength and failure strain were lower in IL-1 treated cartilage after 3 but not 1 week of culture. The superficial and middle layers were most affected by IL-1 induced matrix alteration.
    b. IL-1 induced a significant release of GAG into culture medium and loss of GAG from cartilage explants during all 3 weeks of culture.
    c. Collagen fragmentation products including COL2-3/4$C_{short}$ and COL2-3/4M and hydroxyproline were released into culture medium during weeks 2 and 3 of culture. After 3 weeks of culture there was an increase in the percentage of degraded collagen and the amount of COL2-3/4$C_{short}$ epitope in cartilage explants, in superficial and middle layers corresponding to decreases in tensile strength.

## 6.2 Discussion

Results of Chapter 1 of this dissertation implicate surface alteration as having a causal role in the tensile weakening of macroscopically normal adult articular cartilage with age. It is, as yet, unknown what initiates cartilage surface alteration. It has been postulated to be due to due to adhesive wear, abrasive wear, or fatigue failure [26, 34]. Morphological observations of fibrillation have been made by light and transmission electron microscopy of histological sections [7, 25] as well as by scanning electron microscopy of surface and oblique views [6, 23, 30, 36]. The application of India ink to the articular surface has allowed viewing of the articular surface *in situ* and made areas of mild surface wear more apparent [3, 4, 24]. In this dissertation, digital imaging was applied to quantify the intensity of ink-staining (mean reflectance score) as well as give an index of the surface roughness (variance of the reflectance score). This detailed characterization of the morphology of articular cartilage fibrillation has laid a foundation for further study of the causal mechanisms resulting in disruption of the articular surface, as any experimentally-induced manipulations of the surface should be consistent with these observations.

The loss of sulfated GAG in the deep zone of articular cartilage appears to play a role in the early age-associated progression of cartilage degeneration. This loss of fixed charge density may reflect either a net loss of GAG molecules or alteration of GAG chains. Specific alterations of GAG or aggrecan structure occur with skeletal development, aging, and OA disease. Chondroitin sulfate chains, for example, have been shown to have non-reducing terminal disaccharides with variable 4- and 6-sulfated and 4,6 disulfated residues depending on the stage of skeletal development [28] and degree of OA disease [27]. Of the GAG chains attached to aggrecan, the size and number of chondroitin sulfate chains decrease [15], while the size and number of

keratan sulfate chains increase [14, 16] with age, being partially attributable to selective proteolytic degradation of the GAG-rich region of aggrecan [8]. The decrease with age of GAG in the deep layer without additional elevation in fluorescence seen in chapter 2 (Figure 2.2) suggests that substantial matrix turnover occurs. Further detailed study of the changes in GAG seen with age could give insight into the role of GAG loss in early cartilage degeneration, and the importance of its localization in the deep layer as related to changes in subchondral bone which can markedly affect the overlying articular cartilage [13, 29, 35].

The results of this dissertation point to an important role for collagen degradation and remodeling in the biomechanical weakening of articular cartilage with the progression of cartilage degeneration (Chapter 3). The intrinsic fluorescence of cartilage, an index of the extent of nonenzymatic glycation of the collagen network, diminished markedly in cartilage from superficial, middle, and deep zones (Figure 3.3), returning to levels seen in Young adult cartilage (Figure 2.3). An increase in fluorescence with age, indicative of an increase in nonenzymatic glycation products, has been attributed to a lack of collagen turnover [19]. Diminished background fluorescence in osteoarthritic cartilage has been attributed to the activation of chondrocytes resulting in increased collagen synthesis and degradation [10]. Activated chondrocytes synthesize proteoglycan epitope 7D4 and type X collagen [10], indicating that chondrocytes in OA and mildly degenerate cartilage are expressing proteins similar to those of immature cartilage [9]. This could lead to collagen network remodeling with collagen other than type II being synthesized and resulting in an inferior collagen network.

Chondrocyte synthesis and degradation of matrix molecules plays an important role in maintaining matrix homeostasis and may be altered in early cartilage

degeneration. The loss of cells and altered spatial organization may play a causal role in altered matrix metabolism. The organization of cells into clusters parallel to the articular surface has been reported previously for young adult human articular cartilage [32, 33]. A similar organization was reported in a study of rabbit articular cartilage with evidence that chondrocytes have the capacity to communicate with neighboring cells [5]. How cell clustering or the loss of this spatial organization may affect the biosynthetic activity and function of a chondrocyte is still unknown. Further, measures of cell density, whether by analysis of DNA content or cell counting, cannot define the biosynthetic activity, or lack of activity, of chondrocytes. An age-dependent decline in chondrocyte responsiveness to growth factors and a decline in matrix synthesis [1, 2, 11, 12, 18, 22, 31] has been related to telomere shortening, with links to a phenotype associated with cell senescence [20]. Increased proliferative activity in OA chondrocytes may contribute to the accumulation of senescent, growth-arrested chondrocytes in OA cartilage and minimal matrix turnover or repair [21]. It is unknown how this response of chondrocytes varies with site and depth within a knee joint and, further, how it contributes to the advancement of biomechanical weakening and OA disease.

## 6.3 Future Directions

The current work could be expanded in a number of ways. The analysis of cell density in three dimensions has allowed qualitative observation of cell organization. This could be expanded to quantitatively characterize alterations in cell spatial organization with age and early degeneration. New microscopy methods have been developed to achieve this, such as multi-photon fluorescence microscopy and digital

volumetric imaging. Multi-photon fluorescence microscopy allows imaging to greater depths (~3 times more) than conventional confocal laser scanning microscopy and still has the advantage of viewing cells *in situ* without destruction of the tissue sample [37]. Similarly, digital volumetric imaging allows viewing of cartilage in three dimensions and to depths encompassing the full cartilage thickness [17].

Because of the apparent importance of surface wear in the initiation of age-associated tensile weakening, further study of the roles of abrasive and adhesive wear, as well as fatigue in this would be useful. Controlled experiments inducing mechanical wear in a simulator of joint motion could be performed with the goal of defining the loads, pattern of movement, and consistency of lubrication fluid which may induce the patterns of wear seen in this study.

Further study is needed of the alterations of the collagen network in early cartilage degeneration. To study the striking change in intrinsic fluorescence with the onset of early degeneration, detailed quantification of collagen crosslinks such as pyridinoline and pentosidine with depth and site should be measured in age-matched normal, mildly degenerate, and OA tissue. Controlled manipulations of collagen network constituents may also yield important information about the loss of intrinsic fluorescence. Study of the extent of enzymatic matrix degradation needed to reduce intrinsic fluorescence to levels seen in early degeneration may elucidate the role of those enzymes in early cartilage degeneration.

Studies performed here form the foundation of future studies on the age-associated changes in articular cartilage which progress to early cartilage degeneration. The tensile properties of articular cartilage were a sensitive indicator of changes in the cartilage matrix. Tensile softening with age may contribute to the progression of cartilage degeneration through such mechanisms as loss of cells and net

loss of GAG. Further study is needed of the mechanisms and consequences of age-associated tensile weakening.

## 6.4 References


1. Bayliss MT, Osborne D, Woodhouse S, Davidson C: Sulfation of chondroitin sulfate in human articular cartilage. *J Biol Chem* 274:15892-900, 1999.

2. Brand HS, de Koning MH, van Kampen GP, van der Korst JK: Age related changes in the turnover of proteoglycans from explants of bovine articular cartilage. *J Rheumatol* 18:599-605, 1991.

3. Bullough P, Goodfellow J: The significance of the fine structure of articular cartilage. *J Bone Joint Surg Br* 50:852-7, 1968.

4. Chang DG, Iverson EP, Schinagl RM, Sonoda M, Amiel D, Coutts RD, Sah RL: Quantitation and localization of cartilage degeneration following the induction of osteoarthritis in the rabbit knee. *Osteoarthritis Cartilage* 5:357-72, 1997.

5. Chi SS, Rattner JB, Matyas JR: Communication between paired chondrocytes in the superficial zone of articular cartilage. *J Anat* 205:363-70, 2004.

6. Clark JM, Simonian PT: Scanning electron microscopy of "fibrillated" and "malacic" human articular cartilage: technical considerations. *Microsc Res Tech* 37:299-313, 1997.

7. Collins DH. The pathology of articular and spinal disease. London: Arnold; 1949.

8. Dudhia J, Davidson CM, Wells TM, Vynios DH, Hardingham TE, Bayliss MT: Age-related changes in the content of the C-terminal region of aggrecan in human articular cartilage. *Biochem J* 313:933-40, 1996.

9. Gibson G, Lin DL, Francki K, Caterson B, Foster B: Type X collagen is colocalized with a proteoglycan epitope to form distinct morphological structures in bovine growth cartilage. *Bone* 19:307-15, 1996.

10. Gibson GJ, Verner JJ, Nelson FR, Lin DL: Degradation of the cartilage collagen matrix associated with changes in chondrocytes in osteoarthrosis. Assessment by loss of background fluroescence and immunodetection of matrix components. *J Orthop Res* 19:33-42, 2001.

11. Guerne P-A, Blanco F, Kaelin A, Desgeorges A, Lotz M: Growth factor responsiveness of human articular chondrocytes in aging and development. *Arthritis Rheum* 38:960-8, 1995.

12. Hardingham T, Bayliss M: Proteoglycans of articular cartilage: changes in aging and in joint disease. *Sem Arthritis Rheum* 20S:12-33, 1990.

13. Hayami T, Pickarski M, Wesolowski GA, McLane J, Bone A, Destefano J, Rodan GA, Duong le T: The role of subchondral bone remodeling in osteoarthritis: reduction of cartilage degeneration and prevention of osteophyte formation by alendronate in the rat anterior cruciate ligament transection model. *Arthritis Rheum* 50:1193-206, 2004.

14. Hjertquist SO, Lemperg R: Identification and concentration of the glycosaminoglycans of human articular cartilage in relation to age and osteoarthritis. *Calcif Tissue Res* 10:223-37, 1972.

15. Hjertquist SO, Wasteson A: The molecular weight of chondroitin sulfate from human articular cartilage: effects of age and osteoarthritis. *Calc Tiss Res* 10:31-7, 1972.

16. Holmes MWA, Bayliss MT, Muir H: Hyaluronic acid in human articular cartilage: age-related changes in content and size. *Biochem J* 250:435-41, 1988.

17. Jadin KD, Wong BL, Bae WC, Li KW, Williamson AK, Schumacher BL, Price JH, Sah RL: Depth-varying density and organization of chondrocyte in immature and mature bovine articular cartilage assessed by 3-D imaging and analysis. *J Histochem Cytochem* (In press), 2005.

18. Lorenzo P, Bayliss MT, Heinegard D: A novel cartilage protein (CILP) present in the mid-zone of human articular cartilage increases with age. *J Biol Chem* 273:23463-8, 1998.

19. Maroudas A, Palla G, Gilav E: Racemization of aspartic acid in human articular cartilage. *Connect Tissue Res* 28:161-9, 1992.

20. Martin JA, Buckwalter JA: Telomere erosion and senescence in human articular cartilage chondrocytes. *J Gerontol A Biol Sci Med Sci* 56:B172-9, 2001.

21. Martin JA, Buckwalter JA: Human chondrocyte senescence and osteoarthritis. *Biorheology* 39:145-52, 2002.

22. Martin JA, Ellerbroek SM, Buckwalter JA: Age-related decline in chondrocyte response to insulin-like growth factor-I: the role of growth factor binding proteins. *J Orthop Res* 15:491-8, 1997.

23. McCall JG: Scanning electron microscopy of articular surfaces. *Lancet* 2:1194, 1968.

24. Meachim G: Light microscopy of Indian ink preparations of fibrillated cartilage. *Ann Rheum Dis* 31:457-64, 1972.

25. Meachim G, Ghadially FN, Collins DH: Regressive changes in the superficial layer of human articular cartilage. *Ann Rheum Dis* 24:23, 1965.

26. Meachim G, Stockwell RA: The matrix. In: *Adult Articular Cartilage,* ed. by MAR Freeman, Pitman Medical, Tunbridge Wells, England, 1979, 1-66.

27. Plaas AHK, West LA, Wong-Palms S, Nelson FR: Glycosaminoglycan sulfation in osteoarthritis: alterations at the non-reducing termini of chondroitin and dermatan sulfate. *J Biol Chem* 273:12642-9, 1998.

28. Plaas AHK, Wong-Palms S, Roughley PJ, Midura RJ, Hascall VC: Chemical and immunological assay of the nonreducing terminal residues of chondroitin sulfate from human aggrecan. *J Biol Chem* 272:20603-10, 1997.

29. Pullig O, Weseloh G, Ronneberger D, Kakonen S, Swoboda B: Chondrocyte differentiation in human osteoarthritis: expression of osteocalcin in normal and osteoarthritic cartilage and bone. *Calcif Tissue Int* 67:230-40, 2000.

30. Redler I, Zimny ML: Scanning electron microscopy of normal and abnormal articular cartilage and synovium. *J Bone Joint Surg Am* 52:1395-404, 1970.

31. Rosen F, McCabe G, Quach J, Solan J, Terkeltaub R, Seegmiller JE, Lotz M: Differential effects of aging on human chondrocyte responses to transforming growth factor beta: increased pyrophosphate production and decreased cell proliferation. *Arthritis Rheum* 40:1275-81, 1997.

32. Schumacher BL, Su J-L, Lindley KM, Kuettner KE, Cole AA: Horizontally oriented clusters of multiple chondrons in the superficial zone of ankle, but not knee articular cartilage. *Anat Rec* 266:241-8, 2002.

33. Stockwell RA, Meachim G: The chondrocytes. In: *Adult Articular Cartilage,* ed. by MAR Freeman, Pitman Medical, Tunbridge Wells, England, 1979, 69-144.

34. Swanson SAV: Friction, Wear, and Lubrication. In: *Adult Articular Cartilage,* ed. by MAR Freeman, Pitman Medical, Tunbridge Wells, England, 1979, 415-60.

35. Walker GD, Fischer M, Gannon J, Thompson RC, Oegema TR: Expression of type-X collagen in osteoarthritis. *J Orthop Res* 13:4-12, 1995.

36. Walker PS, Sikorski J, Dowson D, Longfield MD, Wright V, Buckley T: Behaviour of synovial fluid on surfaces of articular cartilage. A scanning electron microscope study. *Ann Rheum Dis* 28:1-14, 1969.

37. Wong B, Wallace V, Coleno M, Benton H, Tromberg B: Two-Photon Excitation Laser Scanning Microscopy of Human, Porcine, and Rabbit Nasal Septal Cartilage. *Tissue Engineering* 7:599-606, 2001.

# APPENDIX A

# ANALYSIS OF CARTILAGE SURFACE STRUCTURE AFTER APPLICATION OF INDIA INK

## A.1 Introduction

Wear of the articular surface may be an important factor in the pathogenesis of osteoarthritis. Application of India ink to the articular surface has highlighted areas of cartilage fibrillation and provided morphological views of cartilage degeneration across a joint surface [1, 3]. This method has been used to define semi-quantitative grades of cartilage degeneration [3] and also semi-quantitative grades of intensity of ink-staining and the percentage of the surface area affected [4]. Recently, a method was developed to quantify the intensity of ink-staining on the articular surface through digital imaging of the cartilage surface [2]. Through this method a reflectance score was obtained with 1 being representative of a smooth articular surface and 0 being representative of degenerate cartilage. However, this method only defines the average intensity of staining over an articular surface and not necessarily the roughness of that surface. As an index of the roughness of an articular surface, the variance of the reflectance score could be defined to give an average squared deviation of the reflectance of all pixels in an image from the mean reflectance score.

The objective, here, was to provide supplemental information to that in Chapters 2 and 3 regarding the mean and variance of the reflectance score of

macroscopically normal cartilage of Young, Middle, and Old age groups, as well as age-matched normal (NL) and mildly degenerate cartilage from arthritic (OA) and non-arthritic joints (DGN), and to determine the distinct patterns of variation with age and with degeneration.

## A.2 Methods

Cartilage from donors described in chapters 2 (Young NL, Middle NL, and Old NL; Table 2.1) and 3 (Aged NL, Aged DGN, and Aged OA; Table 3.1) were analyzed for a reflectance score as described previously [2]. Briefly, samples were swabbed with a 1 to 5 dilution of India ink to PBS+PI and digitally imaged. Each image was normalized to professional light (gray #3) and dark (gray #19) gray scale targets (Q13, Eastman Kodak Co., Rochester, NY) which were chosen to approximate the reflectance of normal, non-staining cartilage and fibrillated, maximally ink-stained cartilage. The region encompassing the articular surface was analyzed for the mean and variance of the reflectance score in a sample according to the following equations, respectively:

$$\text{mean} = \frac{\Sigma(X)}{N}$$

$$\text{variance} = \frac{\Sigma(X - \text{mean})^2}{N - 1}$$

where $X$ is the reflectance associated with an individual pixel and $N$ is the total number of pixels.

## A.3 Results

The mean and variance of the reflectance score exhibitied changes in the surface structure of articular cartilage dependent on site, age, and stage of degeneration. The mean and variance of the reflectance score of Young NL, Middle NL, and Old NL samples of the lateral and medial femoral condyle (LFC, MFC) are shown in Figure 2.1b,c. There was evidence for a relatively high mean reflectance score (Figure 2.1b) in macroscopically normal samples of the LFC, with a mild age-associated increase in the variance (Figure 2.1c). In the MFC, there was an age-associated decrease in the mean reflectance score (Figure 2.1b) and relatively high variance in samples of all age groups (Figure 2.1c). This is shown qualitatively in images of the India ink-stained articular surfaces, with faint striations apparent in Young NL and Middle NL samples of the LFC and mottled surfaces in the Old NL samples of the LFC and all samples of the MFC (Figure A.1). Wiremesh plots of the reflectance score over the surface area illustrate the increasing amounts of roughness over the surface in Young NL, Middle NL, and Old NL samples of the LFC and MFC (Figure A.1).

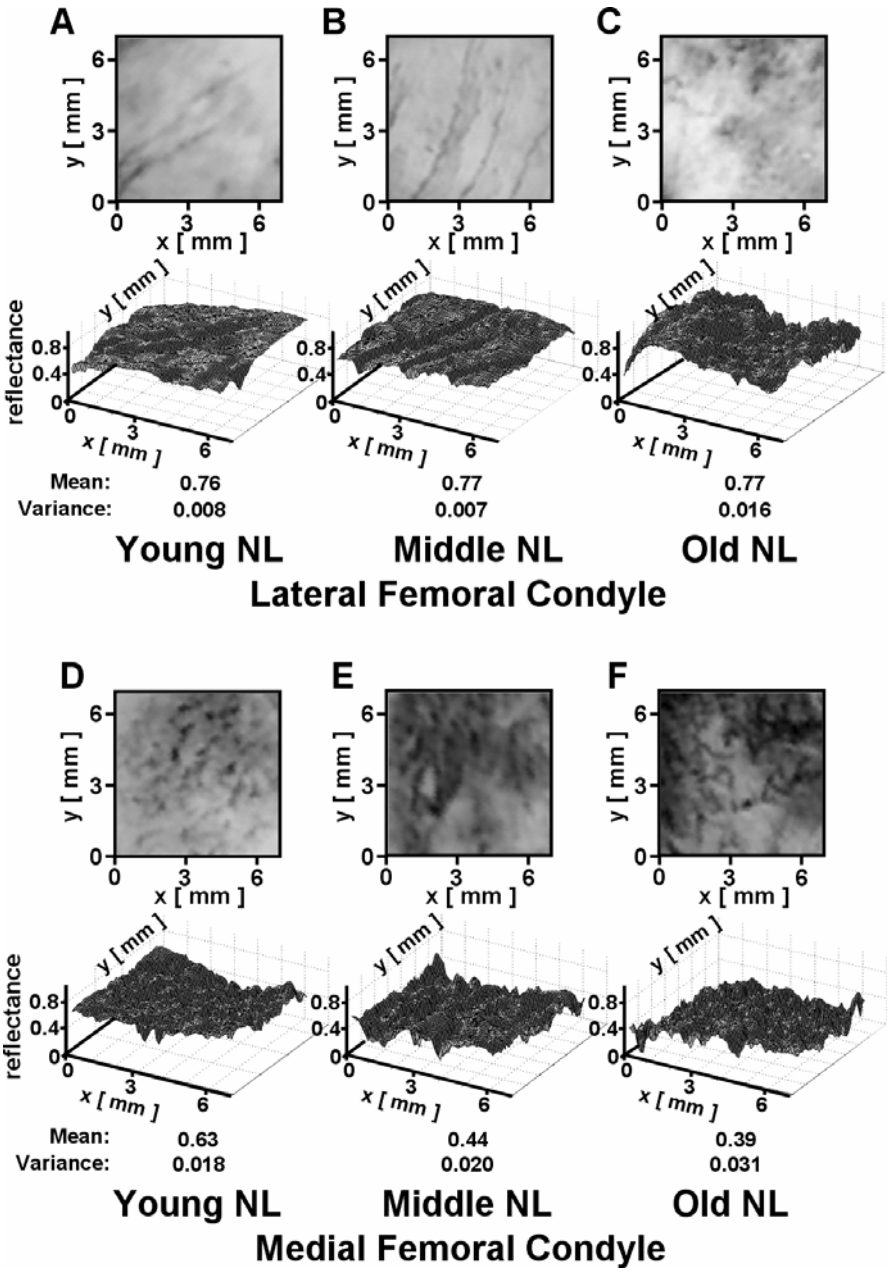



**Figure A.1:** Representative images of ink-stained articular surfaces and wiremesh renderings of cartilage samples. Samples shown had the median reflectance score and were from the lateral femoral condyle (**A**-**C**) and medial femoral condyle (**D**-**F**) of macroscopically normal articular cartilage (NL) of Young (**A**,**D**), Middle (**B**,**E**), and Old (**C**,**F**) age donors described in Table 2.1. Below images of ink-stained surfaces are wiremesh plots, illustrating the light and dark patterns in each sample, as well as the mean and variance of the reflectance score for each sample.

Aged DGN and Aged OA samples showed patterns of fibrillation consistent with early cartilage degeneration. The mean of the reflectance score was low in Aged DGN and Aged OA samples compared to Aged NL samples of the LFC (Figure 3.1b). In the MFC, the mean reflectance score was relatively low in each of the Aged NL, Aged DGN, and Aged OA groups (Figure 3.1b). In parallel with the pattern of lower mean reflectance score in Aged DGN and Aged OA samples of the LFC, the variance of the reflectance score was higher in Aged DGN and Aged OA samples than Aged NL samples (Figure 3.1c). In the MFC, the variance was relatively high in each of the Aged NL, Aged DGN, and Aged OA groups (Figure 3.1c). These surface alterations in Aged NL, Aged DGN and Aged OA samples are observed in images of the ink-stained articular surfaces (Figure A.2). The increase in or relatively high variance in Aged NL, Aged DGN, and Aged OA samples of the LFC and MFC are reflected in wiremesh plots of the reflectance score over the cartilage surface area (Figure A.2).

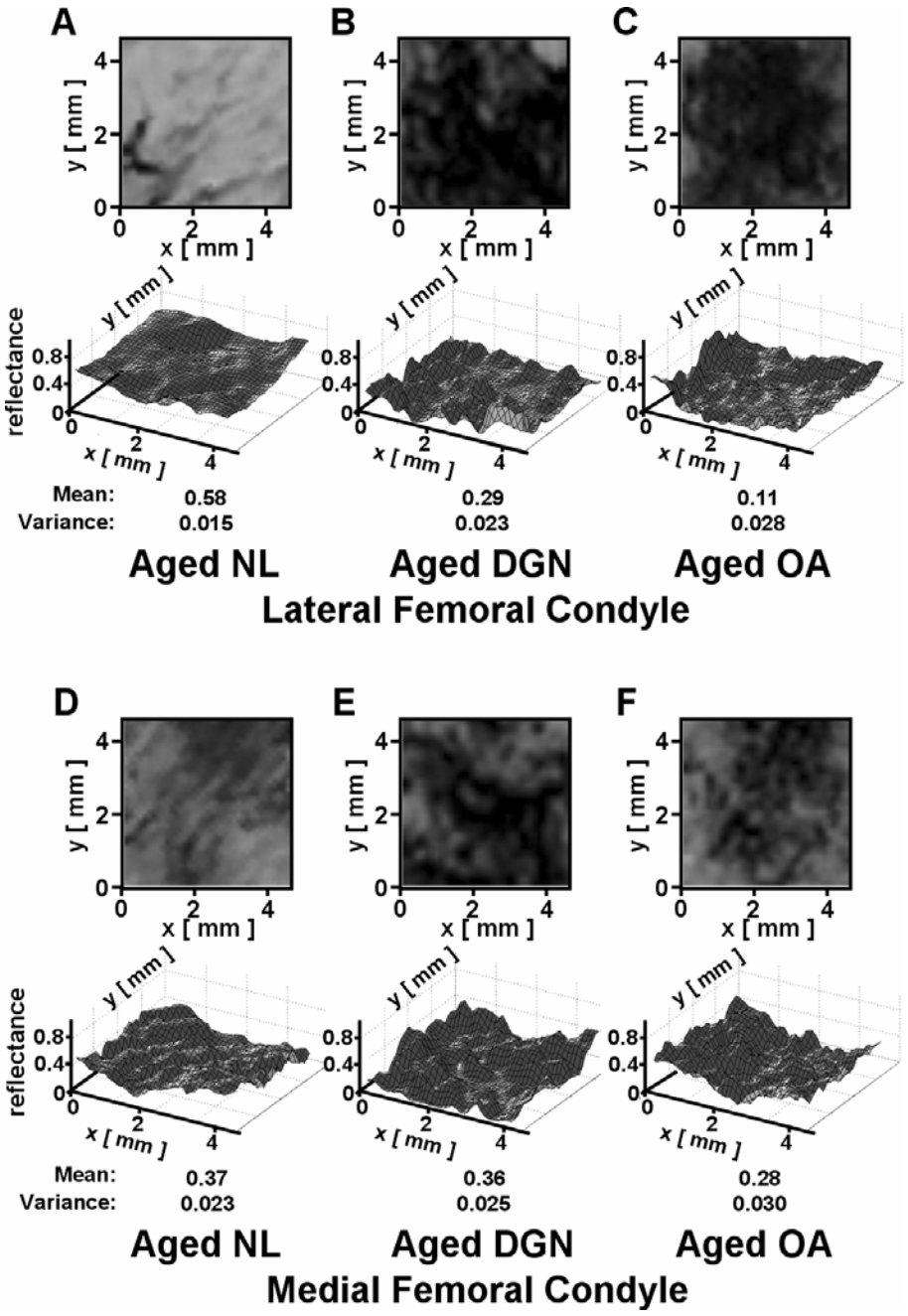



**Figure A.2:** Representative images of ink-stained articular surfaces of cartilage samples. Samples shown had the median reflectance score and were from the lateral femoral condyle (**A**-**C**) and medial femoral condyle (**D**-**F**) of Aged NL (**A**,**D**), Aged DGN (**B**,**E**), and Aged OA (**C**,**F**) donors described in Table 3.1. Below images of ink-stained surfaces are wiremesh plots, illustrating the light and dark patterns in each sample, as well as the mean and variance of the reflectance score for each sample.

## A.4 Discussion

The surface alterations with age and with the onset of early cartilage degeneration were highlighted by measures of the mean and variance of the reflectance score. Previous analyses of cartilage surface degeneration in human articular cartilage used the application of India ink to highlight regions of degeneration and give either qualitative or semi-quantitative measures of ink-staining intensity, which were useful for describing degeneration across an entire joint surface [1, 3, 4]. This application of India ink was used for measuring the percentage of the joint surface area that was affected by cartilage degeneration, and showed that the area of ink-staining increased with age in the patellofemoral articulation [4]. This increase in ink-staining could have been a combination of mild age-associated surface alterations and extensive cartilage damage associated with the onset of early osteoarthritis, similar to those shown above (Figure A.1-2). The combined use of India ink-staining and digital image analysis have allowed for more sensitive quantitation of articular cartilage surface damage.

The pattern of change in surface wear with age in macroscopically normal cartilage showed that surface alterations were site-dependent. In particular, there were earlier mild degenerative changes in the MFC than in the LFC. This is reflected in the mean reflectance score as a decrease from Young to Middle age in the MFC, while the reflectance remained high in the LFC (Figure 2.1b). Similarly, while the variance was already high by Young age in the MFC, there were mild increases in the variance from Young to Middle and Middle to Old age in the LFC (Figure 2.1c). These changes are illustrated in the wiremesh plots of ink-stained cartilage surfaces, with plots of Old NL samples of the LFC and Young, Middle, and Old NL samples of the MFC displaying more and higher magnitude hills and valleys representative of light and dark regions

of the ink-stained cartilage surface (Figure A.1). These changes in the extent and pattern of surface wear coincide with mild age-associated tensile weakening of the LFC and an already weak MFC (Figure 2.2).

The patterns of change in surface wear in age-matched samples with the onset of early cartilage degeneration showed a similar site-dependence, with degenerative changes appearing earlier in the MFC than the LFC (Figure 3.1b, c). This could be evidenced as an increase in the unevenness of the wiremesh plots from Aged NL to Aged DGN and from Aged DGN to Aged OA in the LFC and equally rough wiremesh surfaces in the Aged NL, Aged DGN, and Aged OA samples of the MFC (Figure A.2). These, too, coincide with an ~70% decrease in the tensile strength and stiffness of the superficial layer of articular cartilage seen in Figure 2.2. Surface wear may be an important factor in both age- and degeneration-associated tensile weakening of the articular surface and the progression of early osteoarthritis.

## A.5 References

1. Bullough P, Goodfellow J: The significance of the fine structure of articular cartilage. *J Bone Joint Surg Br* 50:852-7, 1968.

2. Chang DG, Iverson EP, Schinagl RM, Sonoda M, Amiel D, Coutts RD, Sah RL: Quantitation and localization of cartilage degeneration following the induction of osteoarthritis in the rabbit knee. *Osteoarthritis Cartilage* 5:357-72, 1997.

3. Meachim G: Light microscopy of Indian ink preparations of fibrillated cartilage. *Ann Rheum Dis* 31:457-64, 1972.

4. Meachim G, Emery IH: Quantitative aspects of patello-femoral cartilage fibrillation in Liverpool necropsies. *Ann Rheum Dis* 33:39-47, 1974.

# APPENDIX B

# GLYCOSAMINOGLYCAN AND DNA CONTENT RELATIVE TO COLLAGEN

## B.1 Introduction

Because the equilibrium water content of articular cartilage can increase with the onset of early cartilage degeneration, the normalization of biochemical properties to the tissue wet weight can be skewed. The objective, here, was to provide supplemental information to that in Chapters 2 and 3 regarding DNA and glycosaminoglycan (GAG) content of macroscopically normal cartilage of Young, Middle, and Old age groups, as well as age-matched normal and mildly degenerate cartilage from arthritic and non-arthritic joints relative to the collagen (COL) content.

## B.2 Methods

Cartilage from donors described in chapters 2 (Young NL, Middle NL, and Old NL; Table 2.1) and 3 (Aged NL, Aged DGN, and Aged OA; Table 3.1) were analyzed for DNA, GAG, and COL content as described previously. DNA and GAG contents of cartilage samples were normalized to the COL content.

## B.3 Results

DNA and GAG content normalized to the amount of COL in the tissue varied in a manner dependent on site (LFC, MFC) and depth from the articular surface (superficial, middle, deep). Both the DNA per COL and GAG per COL were dependent on the site in the joint (each $p<0.025$) and depth from the articular surface (each $p<0.005$) with DNA per COL being ~200% higher and GAG per COL being ~75% lower in the superficial than the deep layer (Figure B.1). DNA per COL did not vary with age group in the LFC ($p=0.2$-$0.9$). DNA per COL was mildly lower in the Middle than the Young age group in the superficial layer of the MFC ($p=0.15$), but did not vary with age group in middle or deep layers of the MFC ($p=0.4$-$0.9$, Figure B.1a). Changes in GAG per COL were localized to the middle and deep layers of the LFC (Figure B.1b). GAG per COL was lower in the Old age group than the Young age group in the middle and deep layers of the LFC ($p<0.05$ and $p<0.005$, respectively). There was no difference between age groups in the superficial layer of the LFC or at any depth in the MFC ($p=0.2$-$1.0$).

The contents of DNA and GAG normalized to the COL content were similar between age-matched NL, DGN, and OA groups. They did not vary with LFC or MFC site ($p=0.1$ and $p=0.5$, respectively) although they did vary with depth (each $p<0.005$). The DNA per COL was ~120% higher and the GAG per COL was ~66% lower in the superficial layer than the deep layer. There was no difference between age group for DNA per COL or GAG per COL at either the LFC or MFC sites in superficial, middle or deep layers ($0.1$-$1.0$, Figure B.2).

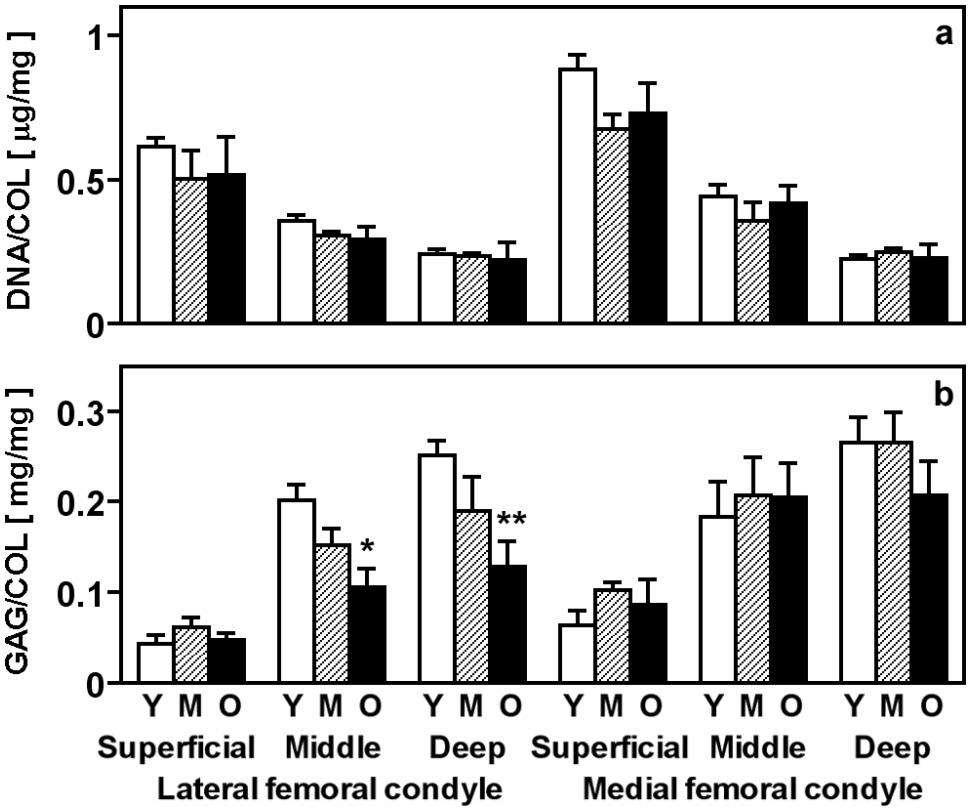



**Figure B.1:** DNA and GAG content determined relative to the COL content of macroscopically normal human articular cartilage samples of Young (Y), Middle (M), and Old (O) age groups described in Table 2.1. Cartilage tissue adjacent to the mechanical test specimens was analyzed for DNA (**a**) and GAG (**b**) in superficial, middle and deep layers. DNA and GAG were each normalized to COL. *n*=9-12. *p<0.05, **p<0.005 versus Young age samples.

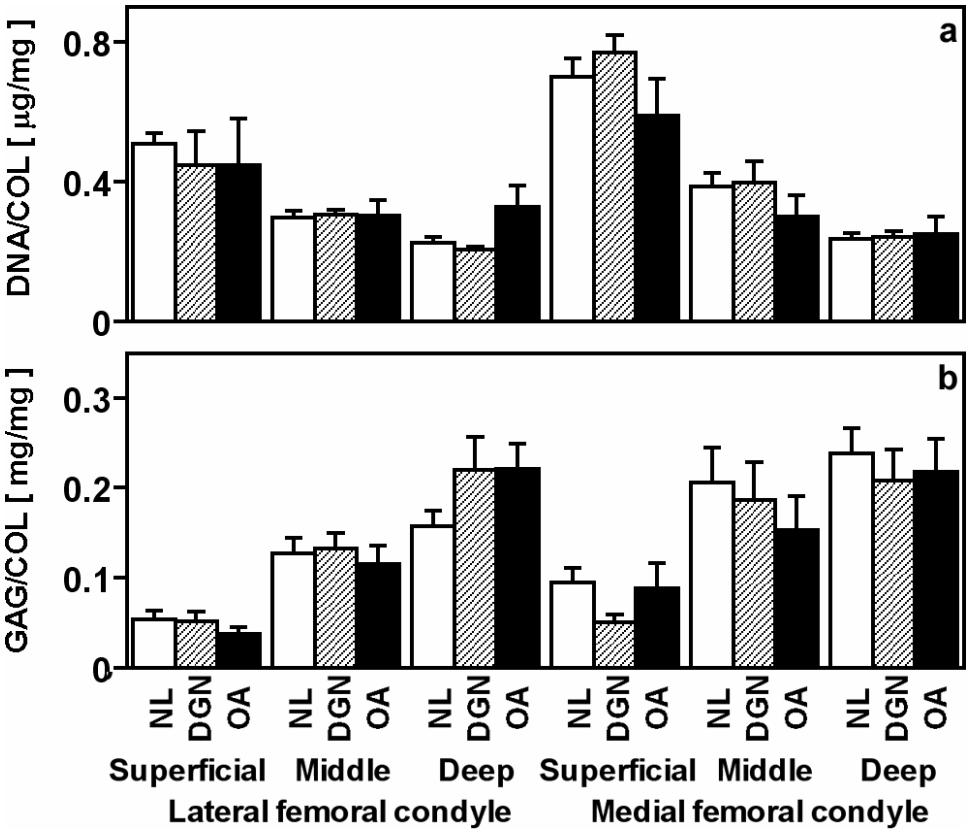



**Figure B.2:** DNA and GAG content determined relative to the COL content of age-matched human articular cartilage samples described in Table 3.1. Cartilage tissue adjacent to the mechanical test specimens was analyzed for DNA (**a**) and GAG (**b**). DNA and GAG were each normalized to COL. *n*=6-21.

## B.4 Discussion

Variations in matrix constituents can depend on the wet and dry weights of the tissue. Because tissue swelling occurs in osteoarthritic cartilage, it may be difficult to interpret results when data are normalized to the tissue volume or wet weight. As is indicated by the results in Chapter 2, tissue swelling does not occur to a significant extent in macroscopically normal cartilage. However, as is indicated in Chapter 3, tissue swelling does occur in OA tissue and can lead to lower estimates of certain matrix constituents, which was the case here in estimates of DNA content (Figures 3.3b, B.2a).

Tissue swelling occurs when osmotically active proteoglycans swell within a degraded collagen network [2, 4]. An increase in the equilibrium water content can have detrimental effects on the cartilage matrix. An increase in the water content correlates negatively with the equilibrium compressive modulus and positively with the hydraulic permeability of cartilage [1, 3] and can adversely affect the way cartilage responds to compressive load. Tissue swelling seen in this work (Figure 3.3a) coincides with an increase in collagen degradation, thus having a detrimental effect on cartilage tensile properties as well.

## B.5 References

1. Armstrong CG, Mow VC: Variations in the intrinsic mechanical properties of human articular cartilage with age, degeneration, and water content. *J Bone Joint Surg Am* 64-A:88-94, 1982.

2. Bank RA, Soudry M, Maroudas A, Mizrahi J, TeKoppele JM: The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. *Arthritis Rheum* 43:2202-10, 2000.

3. Hoch DH, Grodzinsky AJ, Koob TJ, Albert ML, Eyre DR: Early changes in material properties of rabbit articular cartilage after meniscectomy. *J Orthop Res* 1:4-12, 1983.

4. Maroudas A: Balance between swelling pressure and collagen tension in normal and degenerate cartilage. *Nature* 260:808-9, 1976.

# APPENDIX C

# ANALYSIS OF CHANGE IN INTRINSIC FLUORESCENCE OF HUMAN ARTICULAR CARTILAGE WITH AGE AND DEGENERATION

## C.1 Introduction

The tensile weakening of articular cartilage with age and the onset of early cartilage degeneration has been attributed to changes in the collagen network [1, 2, 9, 10]. In particular, changes in matrix constituents in osteoarthritic (OA) cartilage have been associated with collagen turnover and a change of chondrocyte phenotype to that of immature chondrocytes [5, 6]. The detection of collagen denaturation and cleavage epitopes in OA cartilage [4, 7, 8] confirm the alteration of the type II collagen network. Further network remodeling may be measured as an alteration of intrinsic cartilage fluorescence [6].

The intrinsic fluorescence of human articular cartilage results from the presence of products of collagen glycation or advanced glycation end products (AGE). These products have been detected fluorescently and the AGE, pentosidine, is a crosslink characterized in articular cartilage to increase with age after skeletal maturity, in a manner that has been attributed to a lack of collagen turnover [11]. One particular study has localized regions of diminished background fluorescence in osteoarthritic cartilage in a region just below the articular surface which extends to

deeper regions as collagen synthesis and degradation progress [6]. This has been attributed to the activation of chondrocytes resulting in increased collagen synthesis and degradation [6].

The hypothesis of this study was that there was a pattern of variation in the intrinsic fluorescence of macroscopically normal cartilage with age that was distinctly different from that of cartilage demonstrating signs of early cartilage degeneration from arthritic and non-arthritic joints. Thus, the objective was to measure the intrinsic fluorescence spectra of macroscopically normal cartilage of Young, Middle, and Old age groups, as well as age-matched normal and mildly degenerate cartilage from arthritic and non-arthritic joints, and to determine the distinct patterns of variation with age and with degeneration.

## C.2 Methods

Cartilage was obtained from portions of samples described in chapters 2 (Young NL, Middle NL, and Old NL; Table 2.1) and 3 (Aged NL, Aged DGN, and Aged OA; Table 3.1). Portions of cartilage from superficial, middle, and deep layers and solubilized with proteinase K were diluted to a concentration of 0.6 mg wet weight/ml in TE (10 mM Tris-HCl, 1 mM $Na_2EDTA$, pH 7.4). Spectral scans at excitation (Ex) wavelengths of 270-340 nm and emission (Em) wavelengths of 270-500 nm were made in disposable acrylic cuvettes (Sarstedt Inc., Newton, NC) using a fluorescence spectrophotometer (F-2000 Fluorescence Spectrophotometer, Hitachi, San Jose, CA). Fluorescence was normalized to the peak fluorescence at Ex 280/Em 350. These data were analyzed previously (Chapters 2 and 3) for the ratio of pentosidine-associated fluorescence to pyridinoline associated fluorescence.

## C.3 Results

Local fluorescence maxima centered at Ex 275/Em 305 and Ex 280/Em 350 were evident. These peaks appeared consistently in all sample groups (Figures B.1-4), in superficial, middle, and deep layers and at the lateral and medial sites. The latter peak was near the maximum fluorescence of the crosslink pyridinoline (Ex 295/Em 395), a fluorophore of articular cartilage associated with cartilage maturation [3].

A peak with ~30% of the maximum fluorescence of the above peaks was centered at (Ex 325/Em 395), close to the maximum fluorescence of pentosidine (Ex 335/Em 385), a fluorophore which increases with age after skeletal maturity [3]. The pattern of age-associated change in fluorescence at the pentosidine peak was distinctly different from the pattern of degeneration-associated change. At that peak, the normalized fluorescence increased with age group. In the LFC, the fluorescence was ~33% higher in Middle NL than Young NL samples and was ~25% higher in Old NL than Middle NL samples (Figure B.1). In the MFC, the pattern of increased fluorescence at that peak was not as pronounced. In the superficial layer, the fluorescence was ~20% higher in Middle NL than Young NL samples, and ~20% higher in Old NL than Middle NL samples (Figure B.2). In the middle and deep layers, the fluorescence tended to be ~10% higher in Middle NL than Young NL samples and ~10% higher in Old NL than Middle NL samples.

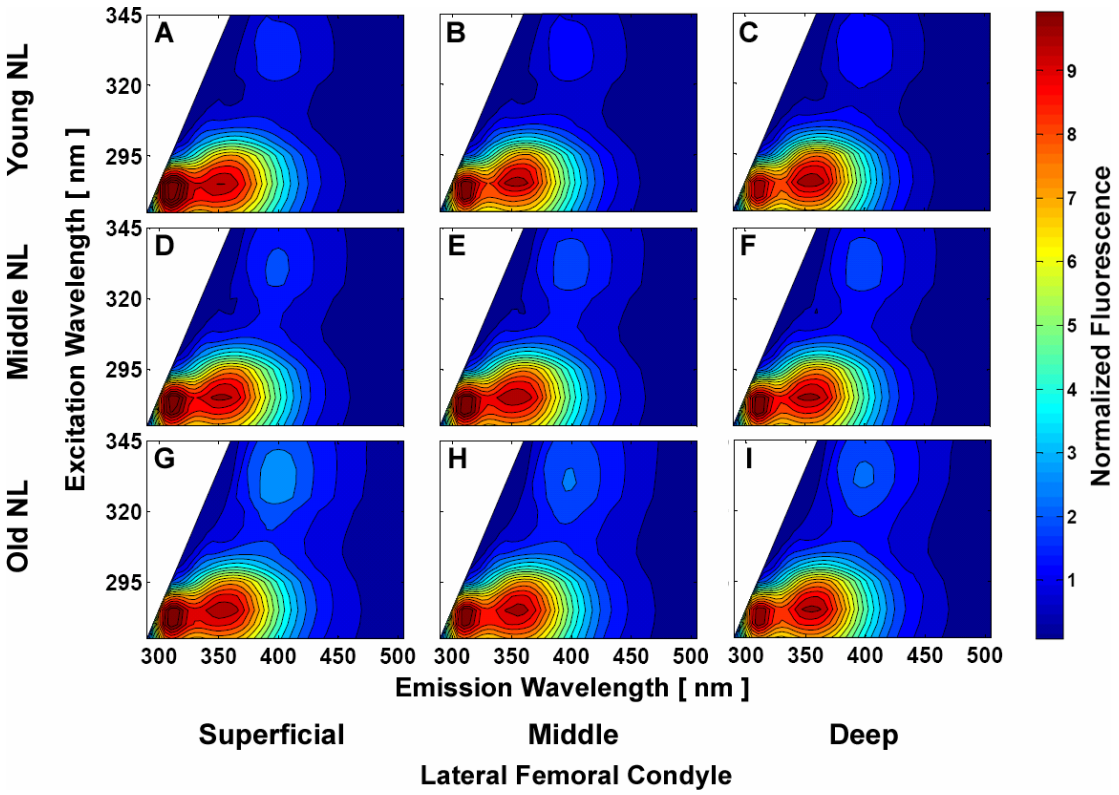



**Figure C.1:** Intrinsic fluorescence spectra of macroscopically normal (NL) articular cartilage of the lateral femoral condyle. Spectral scans at Ex 270-340 nm and Em 270-500 nm were made of solubilized cartilage from Young NL (21-39 yrs old, **A**, **B**, **C**), Middle NL (40-59 yrs old, **D**, **E**, **F**), and Old NL (≥60 yrs old, **G**, **H**, **I**) samples and in superficial (**A**, **D**, **G**), middle (**B**, **E**, **H**), and deep (**C**, **F**, **I**) layers of cartilage.

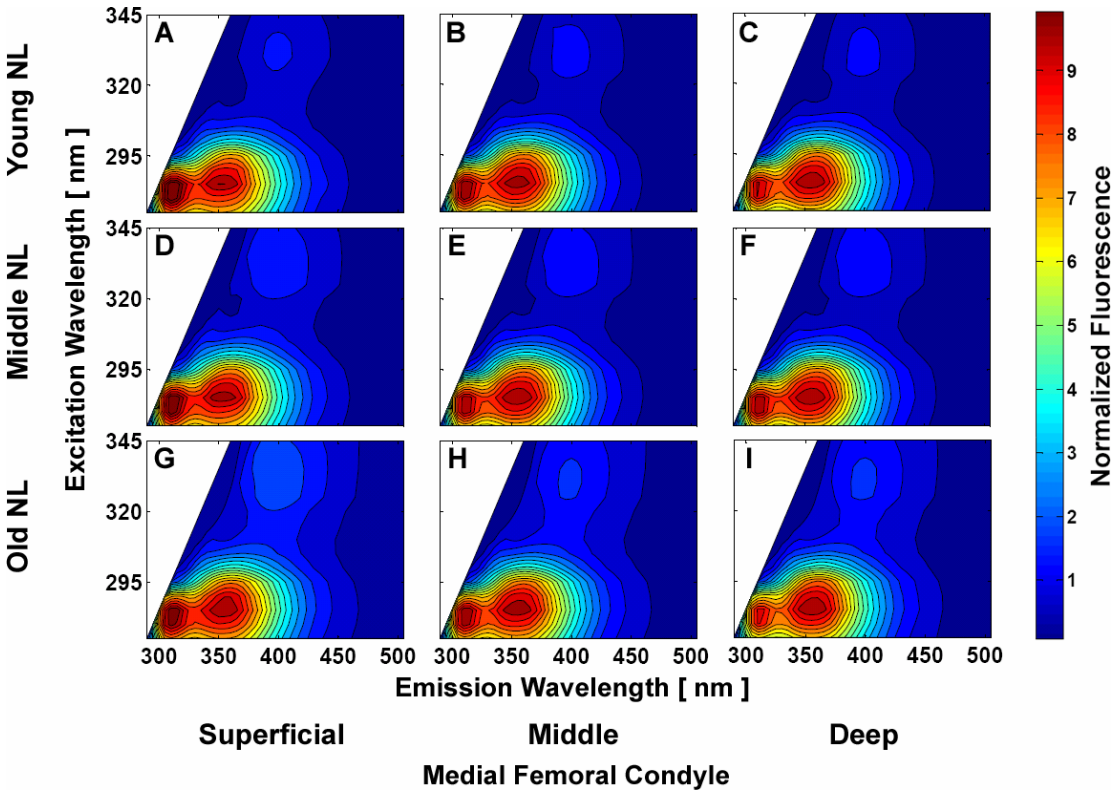



**Figure C.2:** Intrinsic fluorescence spectra of macroscopically normal (NL) articular cartilage of the medial femoral condyle. Spectral scans were made as described in Figure A.1 of cartilage from Young NL (**A**, **B**, **C**), Middle NL (**D**, **E**, **F**), and Old NL (**G**, **H**, **I**) samples and in superficial (**A**, **D**, **G**), middle (**B**, **E**, **H**), and deep (**C**, **F**, **I**) layers of cartilage.

In Aged DGN and Aged OA samples the normalized fluorescence at the pentosidine peak was low compared to Aged NL samples, returning to levels of Young NL samples. In the LFC, the fluorescence was ~30% lower in Aged DGN and Aged OA samples than Aged NL samples in all layers (Figure B.3). In the MFC, where the age-associated increase in normalized fluorescence did not reach similar levels to those in the LFC, the decrease in fluorescence was less pronounced. In the superficial layer, the fluorescence was ~25% lower in Aged DGN and Aged OA samples than in Aged NL samples (Figure B.4). In middle and deep layers, the fluorescence was ~20% lower in Aged DGN and Aged OA samples than in Aged NL samples. The pattern of change in fluorescence in Young, Middle, and Old NL samples indicates an increase in the age-associated pentosidine fluorescence. In Aged DGN and Aged OA samples there is dramatic decrease in that age-associated fluorescence, which may be associated with collagen degradation and remodeling in mildly degenerate cartilage.

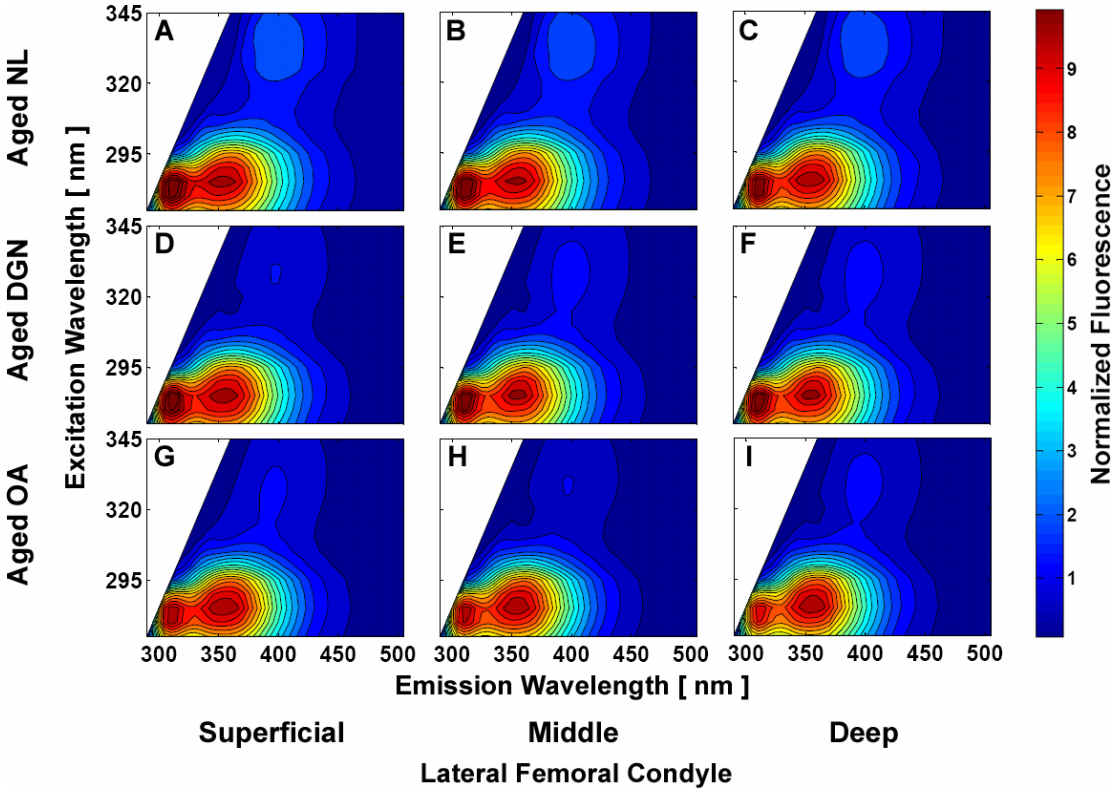



**Figure C.3:** Intrinsic fluorescence spectra of articular cartilage of the lateral femoral condyle. Cartilage was judged to be macroscopically normal (NL) or mildly degenerate cartilage from donors who were (OA) or were not (DGN) diagnosed with osteoarthritis. Spectral scans were made as described in Figure A.1 of cartilage from Aged (≥40 yrs old) NL (**A**, **B**, **C**), DGN (**D**, **E**, **F**), and OA (**G**, **H**, **I**) samples and in superficial (**A**, **D**, **G**), middle (**B**, **E**, **H**), and deep (**C**, **F**, **I**) layers of cartilage.

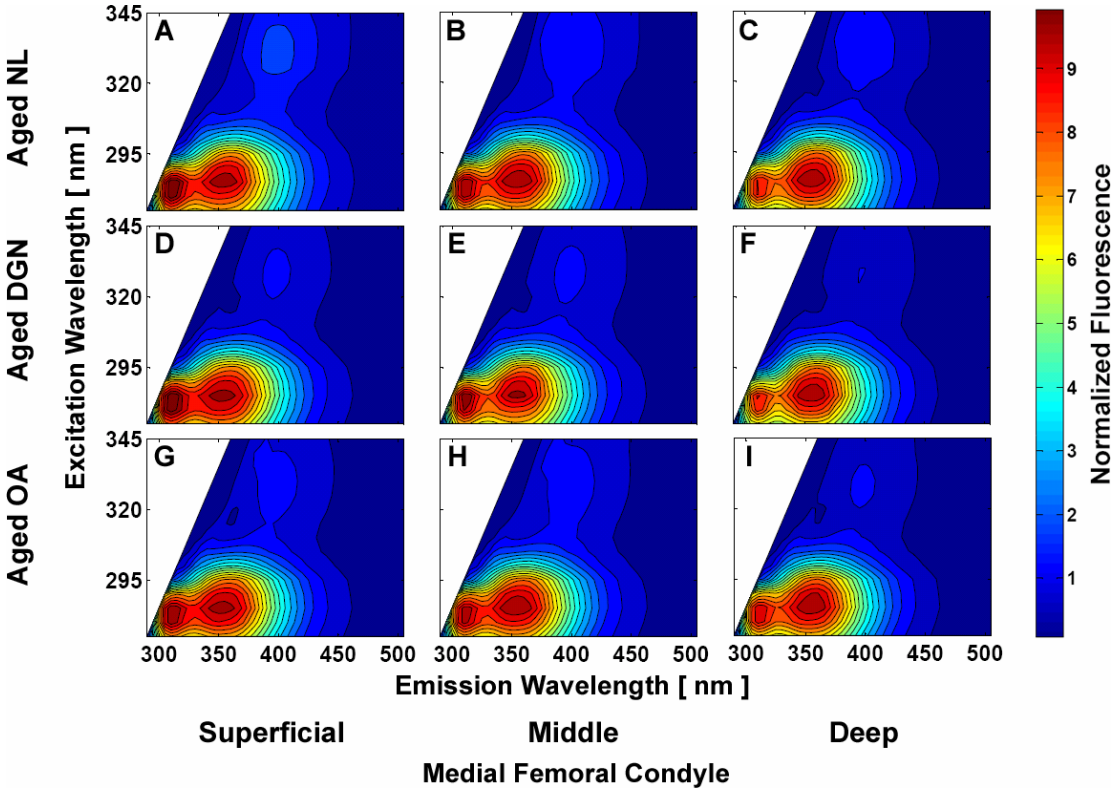



**Figure C.4:** Intrinsic fluorescence spectra of articular cartilage of the medial femoral condyle. Spectral scans were made as described in Figure A.1 of cartilage from Aged NL (**A**, **B**, **C**), DGN (**D**, **E**, **F**), and OA (**G**, **H**, **I**) samples and in superficial (**A**, **D**, **G**), middle (**B**, **E**, **H**), and deep (**C**, **F**, **I**) layers of cartilage.

## C.4 Discussion

The intrinsic fluorescence of articular cartilage is due to the presence of AGEs in articular cartilage. The chemically defined AGE pentosidine, has been shown to increase with age [3] and is attributed to slow or absent collagen turnover [11]. The increase in pentosidine-associated fluorescence with age was confirmed here and the fluorescence was defined at discrete depths (superficial, middle, deep layers) and anatomical locations (LFC, MFC).

Loss of this intrinsic fluorescence has been associated with degradation of the collagen network and activation of articular chondrocytes to synthesize proteoglycan epitope 7D4 and type X collagen [6], which has been suggested to indicate that chondrocytes in OA and mildly degenerate cartilage are expressing proteins similar to those of immature cartilage [5]. The loss of intrinsic fluorescence seen here in mildly degenerate articular cartilage coincides with a decrease in tensile strength and failure strain which occurs in superficial, middle, and deep layers as well as at LFC and MFC sites in Aged DGN and Aged OA compared to Aged NL samples. While collagen degradation has been associated with a loss of tensile integrity, it is unknown what modifications to the collagen network or to the synthesis of collagen network components specifically contribute to the diminished tensile properties of mildly degenerate articular cartilage.

## C.5 References

1. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH: Tensile properties of human knee joint cartilage: I. influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus. *J Orthop Res* 4:379-92, 1986.

2. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Manicourt DH: Tensile properties of human knee joint cartilage: II. correlations between weight bearing and tissue pathology and the kinetics of swelling. *J Orthop Res* 5:173-86, 1987.

3. Bank RA, Bayliss MT, Lafeber FPJG, Maroudas A, TeKoppele JM: Ageing and zonal variation in post-translational modification of collagen in normal human articular cartilage. The age-related increase in non-enzymatic glycation affects biomechanical properties of cartilage. *Biochem J* 330:345-51, 1998.

4. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, Mitchell P, Hambor J, Diekmann O, Tschesche H, Chan J, Van Wart H, Poole AR: Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J Clin Invest* 99:1534-45, 1997.

5. Gibson G, Lin DL, Francki K, Caterson B, Foster B: Type X collagen is colocalized with a proteoglycan epitope to form distinct morphological structures in bovine growth cartilage. *Bone* 19:307-15, 1996.

6. Gibson GJ, Verner JJ, Nelson FR, Lin DL: Degradation of the cartilage collagen matrix associated with changes in chondrocytes in osteoarthrosis. Assessment by loss of background fluroescence and immunodetection of matrix components. *J Orthop Res* 19:33-42, 2001.

7. Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, Poole AR: Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. *J Clin Invest* 93:1722-32, 1994.

8. Hollander AP, Pidoux I, Reiner A, Rorabeck C, Bourne R, Poole AR: Damage to type II collagen in aging and osteoarthritis starts at the articular surface, orginates around chondrocytes, and extends into the cartilage with progressive degeneration. *J Clin Invest* 96:2859-69, 1995.

9. Kempson GE: Relationship between the tensile properties of articular cartilage from the human knee and age. *Ann Rheum Dis* 41:508-11, 1982.

10. Kempson GE, Muir H, Pollard C, Tuke M: The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. *Biochim Biophys Acta* 297:456-72, 1973.

11. Maroudas A, Palla G, Gilav E: Racemization of aspartic acid in human articular cartilage. *Connect Tissue Res* 28:161-9, 1992.

# APPENDIX D

# CORRELATED VARIATIONS OF TENSILE BIOMECHANICAL, BIOCHEMICAL, AND STRUCTURAL PROPERTIES

## D.1 Introduction

The purpose of the following is to study the relationship between tensile properties, indices of surface wear, and biochemical constituents as associated with aging in macroscopically normal articular cartilage (NL) as well as with the onset of early cartilage degeneration (DGN) and osteoarthritis (OA). This is supplemental information to that in Chapters 2 and 3.

## D.2 Methods

Univariate correlation analysis was performed to determine the relationships between tensile properties and biochemical and structural properties. Tests were performed using data for Young NL, Middle NL, and Old NL samples separate from that of Aged NL, Aged DGN, and Aged OA samples to isolate effects of age and degeneration. Relationships between data of the lateral and medial femoral condyles (LFC, MFC) as well as of superficial, middle, and deep layers were tested separately. Variables for testing were chosen based on correspondence with depth with

relationships to indices of surface wear being tested only with properties of the superficial layer.

First, principal component analysis (PCA) was performed to reduce the number of variables to be tested for relationships. Factor 1 parameters with loadings greater than 0.5 were selected based on having generally strong correlations between any two individual components and accounting for the highest percentage of the total variance. Parameters were further narrowed down by selecting relationships between tensile properties and any biochemical or structural property. This was followed by univariate correlation analysis to determine the strength of relationships between these tensile properties and biochemical and structural properties.

## D.3 Results

Results of the PCA analysis on data collected of Young NL, Middle NL, and Old NL samples showed that the donor age, the mean and variance of the reflectance score, tensile ramp modulus and strength, and DNA content were most strongly related (explains ~23% of the total variance) in the superficial layer of the LFC (Table D.1a). In the middle and deep layers of the LFC (Tables D.1b,c) and in the superficial and middle layers of the MFC (Tables D2.a,b), the strongest relationships were between the tensile equilibrium modulus, ramp modulus, and strength, independent of other indices. In the deep layer of the MFC, the strongest relationships were between collagen (COL) content, DNA content, strength, and ramp modulus (explains ~26% of the total variance, Table D.2c). Correlation coefficients and p values for the univariate correlation analyses amongst these variables are given for LFC samples (Tables D.3) and for MFC samples (Tables D.4) and are shown (Figures D.1-5).

Results of the PCA analysis on data collected of Aged NL, DGN, and OA samples showed that the fluorescence ratio, mean reflectance score, histopathology index, tensile equilibrium modulus, ramp modulus, strength, and failure strain were most strongly related (explains ~35% of the total variance) in the superficial layer of the LFC (Table D.5a). In the middle and deep layers of the LFC (Tables D.5b,c) and in the superficial and middle layers of the MFC (Tables D.6a,b), the strongest relationships were between the tensile equilibrium modulus, ramp modulus, and strength, independent of other indices. In the deep layer of the MFC, the strongest relationships were between the ramp modulus, equilibrium modulus, strength, COL content, and DNA content (explains ~35% of the total variance, Table D.6c). Correlation coefficients and p values for the univariate correlation analyses amongst all variables are given for LFC samples (Tables D.7) and for MFC samples (Tables D.8) and are shown (Figures D.6-14).

**a**

| ***Superficial*** | Factor 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Variance of RS | **-0.87** | 0.12 | 0.04 | 0.03 | 0.11 |
| Age | **-0.78** | 0.00 | -0.09 | 0.06 | 0.51 |
| Mean RS | **0.69** | -0.06 | 0.14 | 0.01 | 0.04 |
| Strength | **0.68** | 0.04 | 0.51 | 0.05 | 0.01 |
| DNA Content | **0.55** | 0.34 | -0.35 | 0.44 | -0.01 |
| Ramp Modulus | **0.54** | 0.51 | 0.49 | 0.16 | -0.14 |
| Failure Strain | 0.11 | **-0.83** | -0.28 | 0.03 | 0.20 |
| COL in aCT | 0.02 | **-0.79** | 0.27 | -0.11 | -0.10 |
| Water Content | 0.04 | 0.19 | **-0.87** | -0.22 | 0.14 |
| Equilibrium Modulus | 0.39 | 0.25 | **0.70** | 0.09 | -0.04 |
| COL Content | -0.05 | 0.36 | 0.07 | **0.80** | 0.18 |
| Histopathology Index | -0.17 | 0.31 | -0.32 | **-0.69** | -0.12 |
| GAG Content | -0.14 | 0.02 | 0.18 | **0.59** | -0.57 |
| Fluorescence Ratio | -0.21 | -0.07 | -0.06 | 0.19 | **0.81** |
| Variance Explained [%] | 23 | 15 | 16 | 13 | 10 |

**b**

| ***Middle*** | Factor 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Failure Strain | **-0.92** | 0.10 | 0.01 | 0.09 | 0.08 |
| Equilibrium Modulus | **0.87** | 0.02 | 0.16 | -0.05 | -0.02 |
| Ramp Modulus | **0.86** | 0.33 | 0.10 | 0.27 | 0.14 |
| Age | 0.04 | **-0.85** | 0.34 | 0.04 | 0.03 |
| Strength | 0.17 | **0.79** | 0.28 | 0.30 | -0.15 |
| Fluorescence Ratio | 0.11 | -0.24 | **0.81** | -0.08 | 0.01 |
| DNA Content | 0.05 | 0.16 | **0.72** | -0.01 | 0.11 |
| GAG Content | -0.15 | 0.05 | -0.59 | **-0.71** | -0.10 |
| COL Content | 0.09 | -0.27 | 0.45 | **-0.77** | -0.07 |
| Water Content | -0.10 | -0.21 | 0.03 | 0.18 | **0.91** |
| COL in aCT | -0.30 | -0.40 | -0.32 | 0.31 | **-0.58** |
| Variance Explained [%] | 23 | 17 | 19 | 13 | 11 |

**c**

| ***Deep*** | Factor 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Ramp Modulus | **0.93** | -0.05 | -0.06 | 0.14 |
| Strength | **0.80** | 0.13 | -0.02 | 0.39 |
| Failure Strain | **-0.78** | 0.02 | -0.01 | 0.25 |
| Equilibrium Modulus | **0.65** | 0.64 | 0.17 | -0.13 |
| Water Content | -0.16 | **-0.91** | 0.05 | -0.16 |
| COL Content | -0.21 | **0.70** | 0.17 | 0.51 |
| GAG Content | -0.20 | 0.61 | **-0.62** | -0.20 |
| Age | -0.21 | 0.02 | **0.93** | -0.12 |
| Fluorescence Ratio | 0.17 | 0.15 | **0.60** | 0.44 |
| DNA Content | 0.16 | 0.21 | 0.03 | **0.76** |
| COL in aCT | 0.06 | 0.04 | -0.04 | **-0.73** |
| Variance Explained [%] | 25 | 20 | 15 | 17 |

**Table D.1:** Principal component analysis to identify dominant parameters in Young NL, Middle NL, and Old NL samples of the lateral femoral condyle. Loading coefficients of variation and percent of total variance for each factor are shown for superficial (**a**), middle (**b**), and deep (**c**) layers, with dominant parameters in bold. Indices of surface wear including the mean and variance of the reflectance score (RS) and the histopathology index were correlated with data of the superficial layer.

**a**

| ***Superficial*** | Factor 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| COL Content | **-0.70** | 0.12 | 0.29 | -0.18 | -0.18 |
| DNA Content | **-0.68** | 0.02 | -0.05 | -0.13 | 0.09 |
| Histopathology Index | **0.68** | -0.15 | 0.17 | -0.20 | -0.11 |
| Age | **0.68** | 0.21 | 0.54 | 0.14 | 0.06 |
| Variance of RS | **0.50** | -0.29 | 0.29 | -0.56 | -0.02 |
| Equilibrium Modulus | 0.03 | **0.89** | -0.06 | -0.21 | 0.05 |
| Ramp Modulus | -0.24 | **0.84** | 0.07 | -0.17 | -0.10 |
| Strength | 0.01 | **0.84** | 0.04 | 0.31 | -0.15 |
| Fluorescence Ratio | 0.17 | 0.08 | **0.84** | 0.01 | 0.09 |
| COL in aCT | 0.31 | 0.41 | **-0.66** | -0.07 | 0.36 |
| Failure Strain | 0.27 | -0.29 | 0.19 | **0.83** | 0.10 |
| GAG Content | -0.22 | -0.14 | -0.11 | 0.16 | **0.79** |
| Mean RS | -0.42 | 0.04 | -0.33 | 0.05 | **-0.66** |
| Water Content | 0.27 | -0.47 | 0.32 | 0.27 | -0.43 |
| Variance Explained [%] | 19 | 20 | 14 | 10 | 11 |

**b**

| ***Middle*** | Factor 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Ramp Modulus | **0.93** | 0.09 | -0.01 | 0.03 |
| Equilibrium Modulus | **0.78** | -0.36 | 0.19 | 0.01 |
| Strength | **0.69** | 0.02 | 0.25 | 0.01 |
| Failure Strain | **-0.67** | -0.09 | 0.25 | -0.17 |
| GAG Content | -0.12 | **-0.88** | 0.24 | 0.20 |
| Water Content | -0.46 | **0.73** | 0.16 | 0.15 |
| Fluorescence Ratio | 0.20 | **0.56** | 0.39 | -0.47 |
| COL Content | 0.23 | -0.21 | **0.77** | 0.16 |
| COL in aCT | 0.35 | -0.11 | **-0.75** | 0.27 |
| DNA Content | 0.37 | 0.41 | **0.55** | 0.29 |
| Age | -0.13 | 0.07 | -0.06 | **-0.92** |
| Variance Explained [%] | 27 | 18 | 17 | 12 |

**c**

| ***Deep*** | Factor 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| COL Content | **0.90** | 0.02 | 0.05 | 0.04 |
| Strength | **0.81** | -0.05 | 0.23 | 0.10 |
| DNA Content | **0.69** | 0.08 | -0.29 | 0.39 |
| Ramp Modulus | **0.64** | 0.05 | 0.68 | -0.02 |
| Age | -0.22 | **0.83** | 0.28 | 0.04 |
| COL in aCT | -0.01 | **-0.78** | 0.26 | -0.01 |
| Fluorescence Ratio | 0.50 | **0.64** | -0.04 | -0.10 |
| Failure Strain | 0.09 | 0.11 | **-0.83** | 0.21 |
| Equilibrium Modulus | 0.23 | 0.11 | **0.68** | 0.54 |
| Water Content | -0.39 | -0.12 | 0.08 | **-0.68** |
| GAG Content | -0.22 | -0.49 | -0.05 | **0.65** |
| Variance Explained [%] | 26 | 18 | 17 | 13 |

**Table D.2:** Principal component analysis to identify dominant parameters in Young NL, Middle NL, and Old NL samples of the medial femoral condyle. Loading coefficients of variation and percent of total variance for each factor are shown for superficial (**a**), middle (**b**), and deep (**c**) layers, with dominant parameters in bold.

**a** **Lateral Femoral Condyle**

| ***Superficial*** | Strength | | Equilibrium Modulus | | Ramp Modulus | | Failure Strain | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Age | 0.34 | **0.001** | 0.10 | 0.077 | 0.30 | **0.001** | 0.00 | 0.801 |
| Mean RS | 0.08 | 0.129 | 0.10 | 0.080 | 0.15 | **0.034** | 0.00 | 0.826 |
| Histopathology Index | 0.03 | 0.418 | 0.03 | 0.421 | 0.01 | 0.555 | 0.04 | 0.346 |
| Variance of RS | 0.19 | **0.013** | 0.08 | 0.131 | 0.12 | 0.053 | 0.01 | 0.686 |
| Water Content | 0.13 | 0.067 | 0.26 | **0.008** | 0.14 | 0.061 | 0.01 | 0.666 |
| GAG Content | 0.00 | 0.909 | 0.03 | 0.393 | 0.07 | 0.165 | 0.02 | 0.401 |
| DNA Content | 0.08 | 0.114 | 0.03 | 0.346 | 0.15 | **0.031** | 0.01 | 0.641 |
| COL Content | 0.01 | 0.660 | 0.05 | 0.250 | 0.05 | 0.275 | 0.06 | 0.194 |
| COL in aCT | 0.02 | 0.451 | 0.00 | 0.892 | 0.05 | 0.276 | 0.21 | **0.017** |
| Fluorescence Ratio | 0.03 | 0.391 | 0.05 | 0.240 | 0.02 | 0.426 | 0.03 | 0.347 |

**b**

| ***Middle*** | Strength | | Equilibrium Modulus | | Ramp Modulus | | Failure Strain | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Age | 0.21 | **0.010** | 0.02 | 0.459 | 0.06 | 0.859 | 0.00 | 0.859 |
| Water Content | 0.05 | 0.280 | 0.01 | 0.659 | 0.00 | 0.845 | 0.02 | 0.489 |
| GAG Content | 0.12 | 0.054 | 0.02 | 0.474 | 0.13 | **0.045** | 0.01 | 0.637 |
| DNA Content | 0.03 | 0.384 | 0.02 | 0.473 | 0.05 | 0.251 | 0.01 | 0.523 |
| COL Content | 0.05 | 0.233 | 0.02 | 0.442 | 0.04 | 0.332 | 0.04 | 0.294 |
| COL in aCT | 0.09 | 0.138 | 0.10 | 0.108 | 0.14 | 0.055 | 0.03 | 0.415 |
| Fluorescence Ratio | 0.00 | 0.966 | 0.04 | 0.305 | 0.01 | 0.537 | 0.01 | 0.617 |

**c**

| ***Deep*** | Strength | | Equilibrium Modulus | | Ramp Modulus | | Failure Strain | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Age | 0.04 | 0.281 | 0.00 | 0.748 | 0.07 | 0.140 | 0.00 | 0.768 |
| Water Content | 0.09 | 0.129 | 0.37 | **0.001** | 0.02 | 0.444 | 0.01 | 0.688 |
| GAG Content | 0.03 | 0.364 | 0.03 | 0.357 | 0.03 | 0.353 | 0.00 | 0.786 |
| DNA Content | 0.15 | **0.032** | 0.02 | 0.435 | 0.03 | 0.392 | 0.00 | 0.810 |
| COL Content | 0.02 | 0.519 | 0.06 | 0.227 | 0.03 | 0.384 | 0.03 | 0.420 |
| COL in aCT | 0.02 | 0.502 | 0.00 | 0.896 | 0.00 | 0.944 | 0.01 | 0.551 |
| Fluorescence Ratio | 0.08 | 0.154 | 0.03 | 0.353 | 0.06 | 0.204 | 0.01 | 0.636 |

**Table D.3:** Correlation coefficients of determination ($R^2$) and significance (p) values of univariate linear regression analysis for Young NL, Middle NL, and Old NL samples of the lateral femoral condyle. Values are shown for superficial (**a**), middle (**b**), and deep (**c**) layers, with dominant parameters in bold.

**a** **Medial Femoral Condyle**

| ***Superficial*** | **Strength** | | **Equilibrium Modulus** | | **Ramp Modulus** | | **Failure Strain** | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Age | 0.02 | 0.433 | 0.03 | 0.373 | 0.00 | 0.869 | 0.10 | 0.088 |
| Mean RS | 0.01 | 0.569 | 0.00 | 0.999 | 0.01 | 0.514 | 0.17 | 0.265 |
| Histopathology Index | 0.05 | 0.246 | 0.07 | 0.190 | 0.05 | 0.259 | 0.02 | 0.468 |
| Variance of RS | 0.06 | 0.169 | 0.01 | 0.663 | 0.00 | 0.883 | 0.03 | 0.316 |
| Water Content | 0.02 | 0.555 | 0.28 | **0.008** | 0.03 | 0.419 | 0.12 | 0.099 |
| GAG Content | 0.01 | 0.562 | 0.01 | 0.579 | 0.02 | 0.396 | 0.02 | 0.433 |
| DNA Content | 0.00 | 0.861 | 0.00 | 0.974 | 0.00 | 0.763 | 0.05 | 0.226 |
| COL Content | 0.00 | 0.868 | 0.03 | 0.361 | 0.09 | 0.130 | 0.09 | 0.112 |
| COL in aCT | 0.05 | 0.263 | 0.15 | **0.046** | 0.06 | 0.194 | 0.05 | 0.239 |
| Fluorescence Ratio | 0.00 | 0.834 | 0.00 | 0.769 | 0.00 | 0.904 | 0.02 | 0.453 |

**b**

| ***Middle*** | **Strength** | | **Equilibrium Modulus** | | **Ramp Modulus** | | **Failure Strain** | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Age | 0.07 | 0.147 | 0.01 | 0.576 | 0.04 | 0.255 | 0.02 | 0.455 |
| Water Content | 0.07 | 0.211 | 0.21 | **0.024** | 0.07 | 0.220 | 0.04 | 0.328 |
| GAG Content | 0.02 | 0.402 | 0.04 | 0.286 | 0.08 | 0.123 | 0.06 | 0.193 |
| DNA Content | 0.08 | 0.123 | 0.04 | 0.267 | 0.11 | 0.068 | 0.01 | 0.606 |
| COL Content | 0.01 | 0.714 | 0.18 | **0.025** | 0.00 | 0.934 | 0.03 | 0.391 |
| COL in aCT | 0.07 | 0.169 | 0.02 | 0.440 | 0.08 | 0.157 | 0.08 | 0.155 |
| Fluorescence Ratio | 0.04 | 0.284 | 0.00 | 0.723 | 0.00 | 0.882 | 0.01 | 0.651 |

**c**

| ***Deep*** | **Strength** | | **Equilibrium Modulus** | | **Ramp Modulus** | | **Failure Strain** | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Age | 0.02 | 0.510 | 0.03 | 0.332 | 0.01 | 0.651 | 0.01 | 0.616 |
| Water Content | 0.06 | 0.262 | 0.08 | 0.190 | 0.03 | 0.400 | 0.03 | 0.450 |
| GAG Content | 0.00 | 0.759 | 0.04 | 0.313 | 0.03 | 0.401 | 0.01 | 0.574 |
| DNA Content | 0.15 | **0.035** | 0.01 | 0.565 | 0.08 | 0.148 | 0.06 | 0.201 |
| COL Content | 0.39 | **0.001** | 0.06 | 0.238 | 0.31 | **0.003** | 0.00 | 0.961 |
| COL in aCT | 0.01 | 0.724 | 0.00 | 0.748 | 0.00 | 0.859 | 0.05 | 0.256 |
| Fluorescence Ratio | 0.12 | 0.082 | 0.03 | 0.411 | 0.07 | 0.202 | 0.02 | 0.540 |

**Table D.4:** Correlation coefficients of determination ($R^2$) and significance (p) values of univariate linear regression analysis for Young NL, Middle NL, and Old NL samples of the medial femoral condyle. Values are shown for superficial (**a**), middle (**b**), and deep (**c**) layers, with dominant parameters in bold.

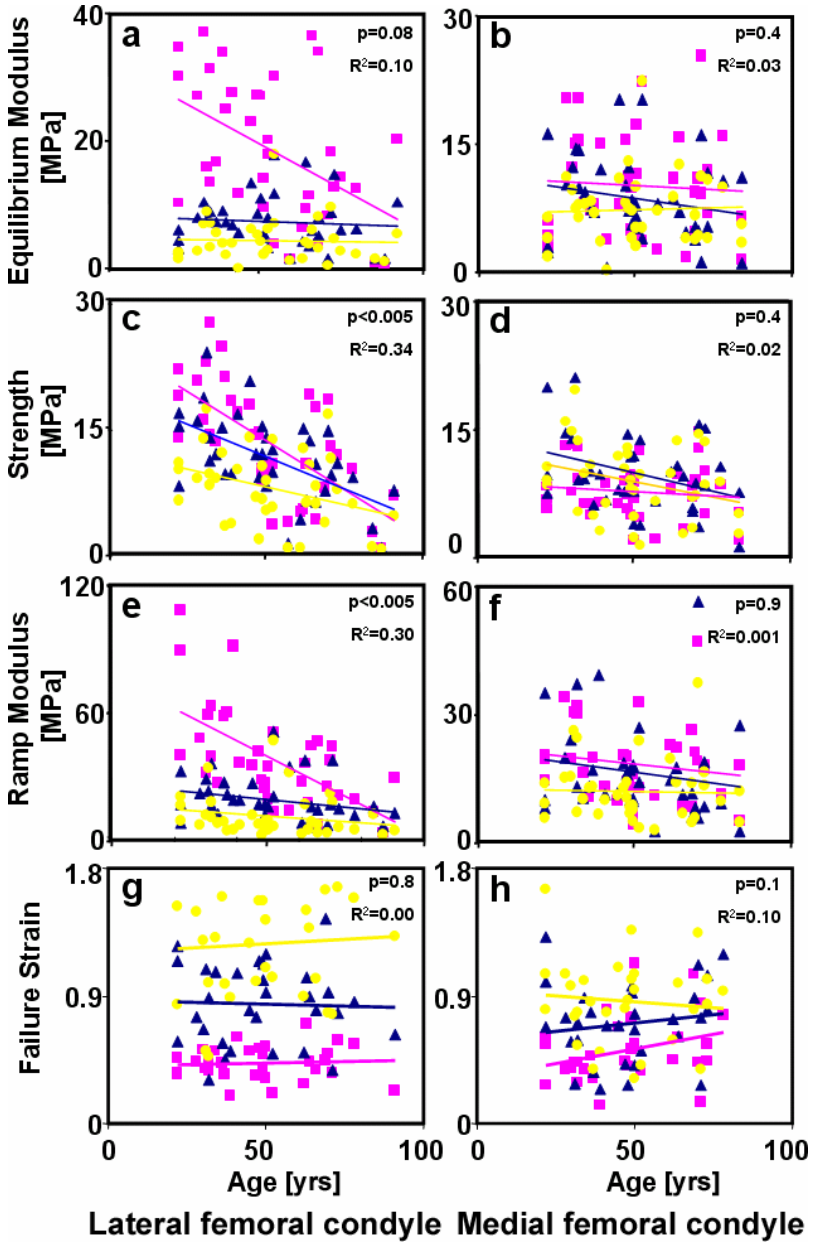



**Figure D.1:** Relationships between tensile properties and age of Young NL, Middle NL, and Old NL samples. Relationships between the tensile equilibrium modulus (**a**, **b**), strength (**c**, **d**), ramp modulus (**e**, **f**), and failure strain (**g**, **h**) and donor age for data from superficial (■), middle (▲), and deep (●) layers of the lateral (**a**, **c**, **e**, **g**) and medial (**b**, **d**, **f**, **h**) femoral condyles are shown. $R^2$ and p-values shown are for the relationship with data of the superficial layer. Lines represent linear regression fits and are meant only to show trends.

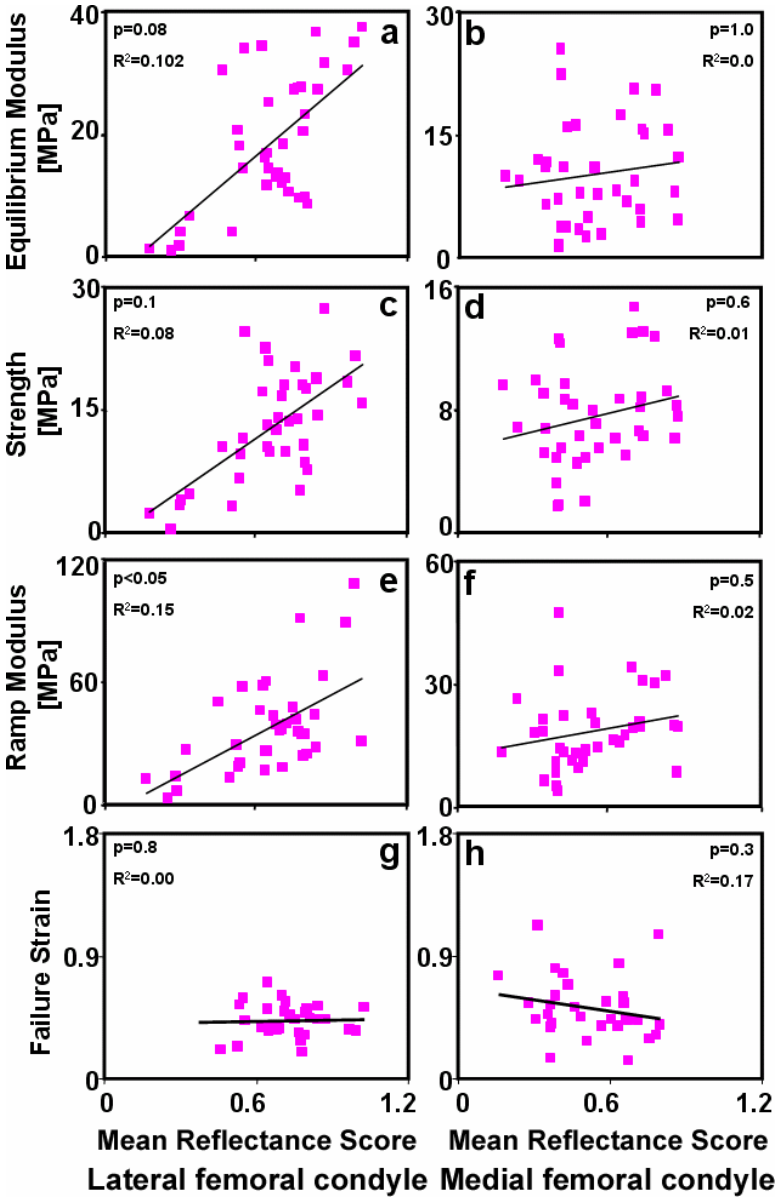



**Figure D.2:** Relationships between tensile properties and mean of the reflectance score of Young NL, Middle NL, and Old NL samples are plotted as described in Figure D.1.

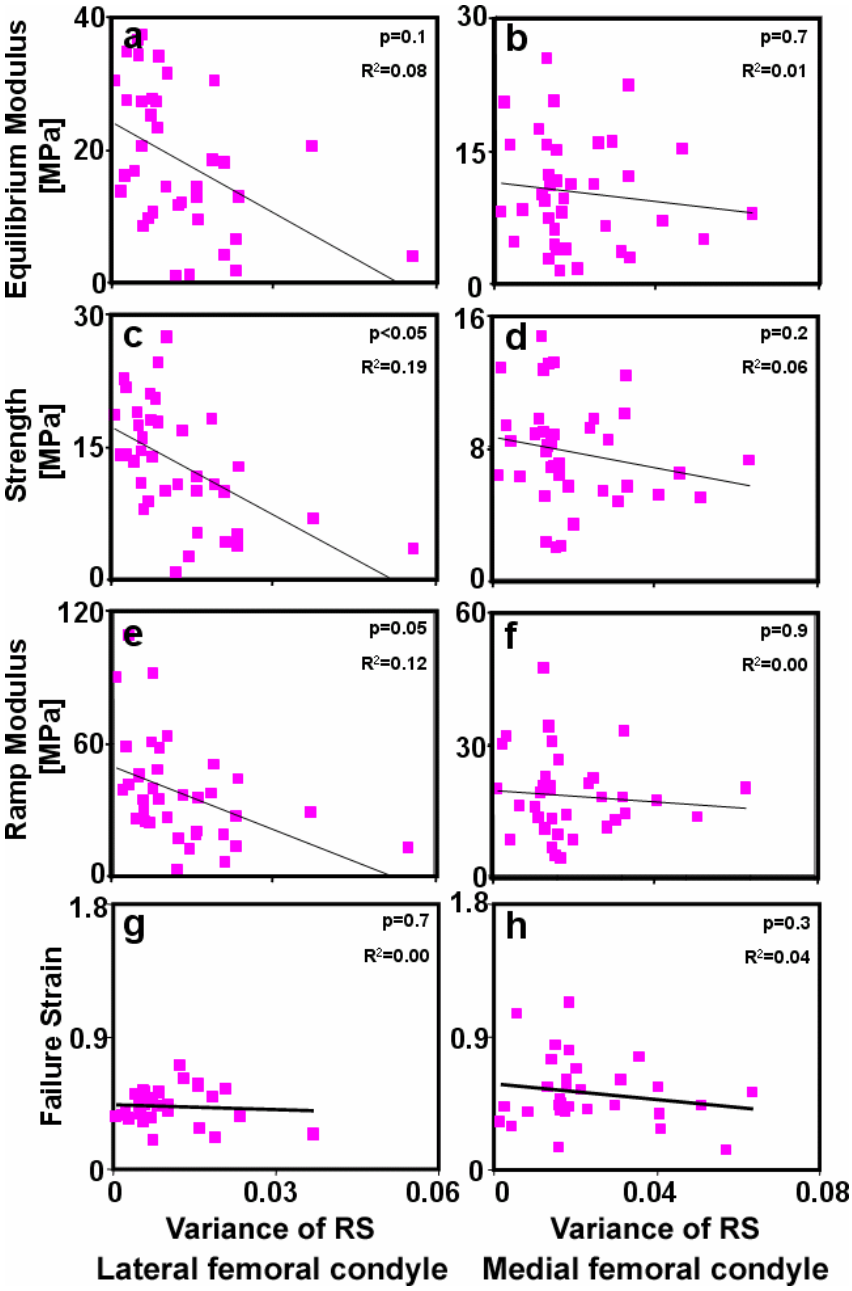



**Figure D.3:** Relationships between tensile properties and variance of the reflectance score of Young NL, Middle NL, and Old NL samples are plotted as described in Figure D.1.

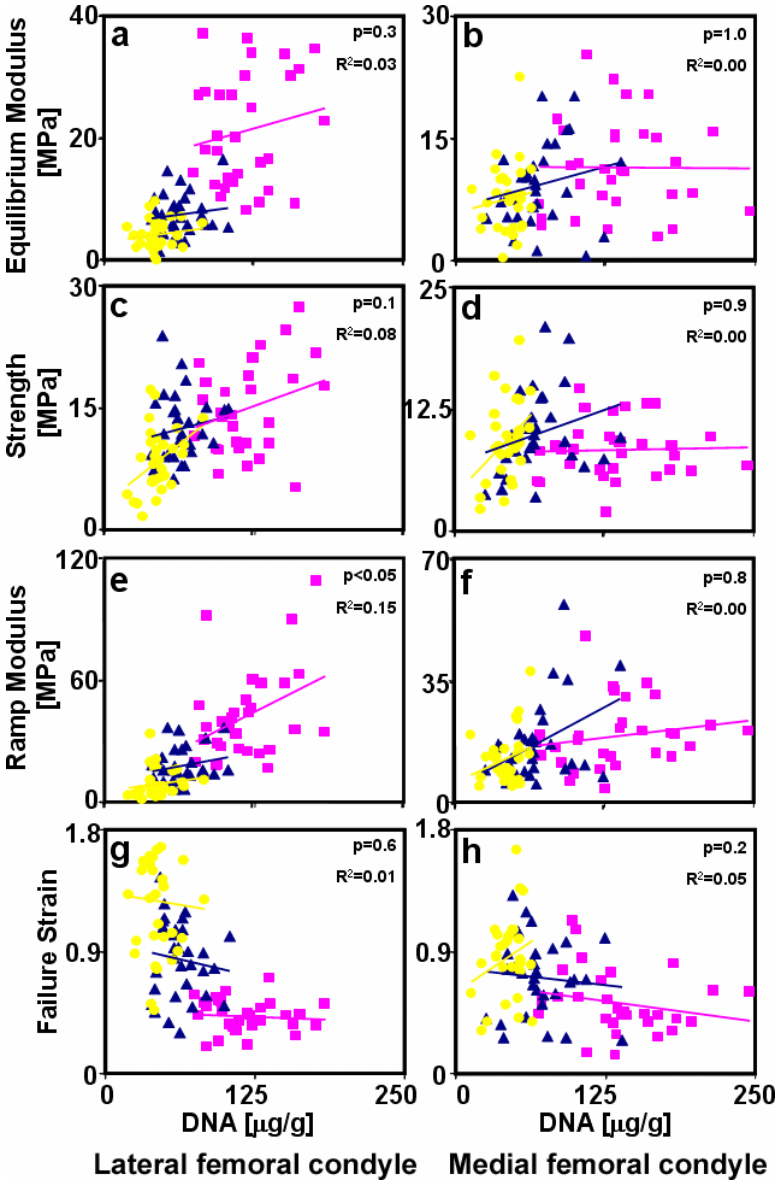



**Figure D.4:** Relationships between tensile properties and DNA content of Young NL, Middle NL, and Old NL samples are plotted as described in Figure D.1.

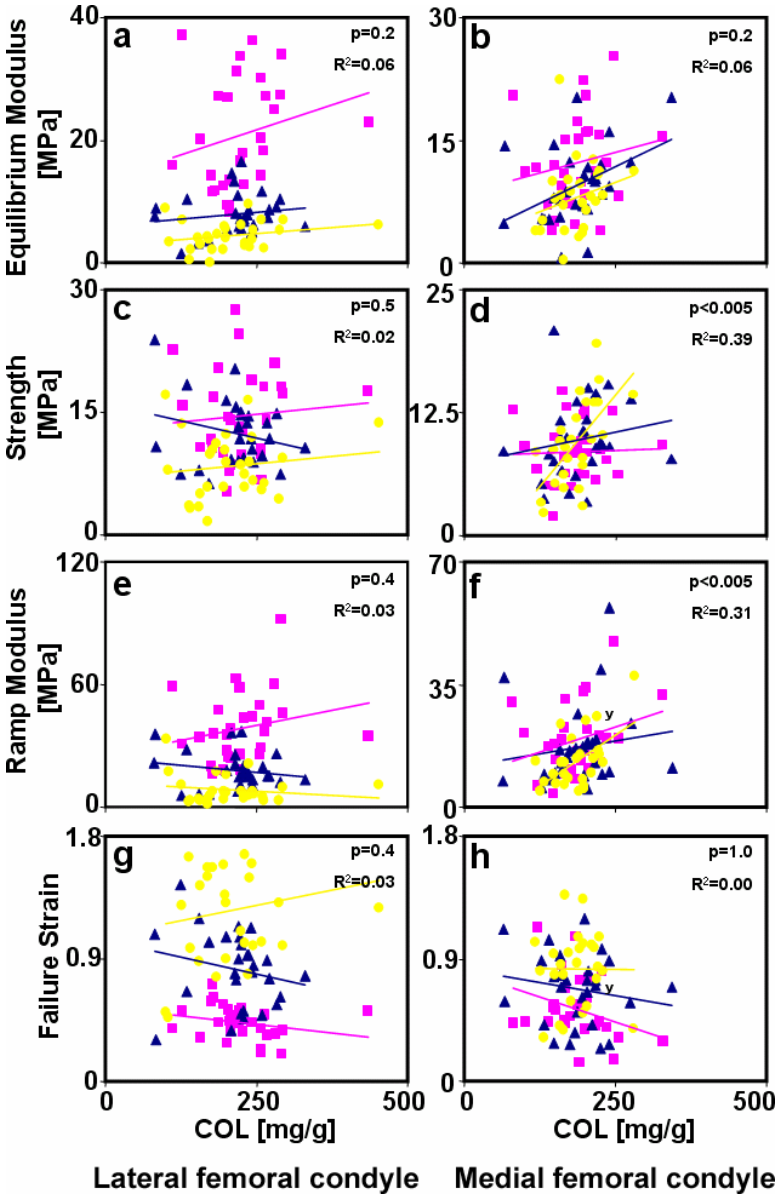



**Figure D.5:** Relationships between tensile properties and collagen (COL) content of Young NL, Middle NL, and Old NL samples are plotted as described in Figure D.1. $R^2$ and p-values shown are for the relationship with data of the deep layer.

**a**

| ***Superficial*** | Factor 1 | 2 | 3 |
|---|---|---|---|
| Failure Strain | **0.90** | -0.18 | 0.12 |
| Fluorescence Ratio | **0.83** | 0.26 | ` |
| Mean RS | **0.78** | 0.43 | 0.18 |
| Histopathology Index | **-0.77** | -0.31 | -0.37 |
| Strength | **0.77** | 0.43 | 0.28 |
| Ramp Modulus | **0.56** | **0.59** | 0.12 |
| Equilibrium Modulus | **0.52** | **0.56** | 0.44 |
| DNA Content | 0.51 | **0.57** | 0.09 |
| COL in aCT | -0.18 | **-0.85** | 0.05 |
| COL Content | 0.02 | **0.80** | 0.18 |
| GAG Content | 0.00 | 0.11 | **0.90** |
| Water Content | -0.46 | -0.03 | **-0.76** |
| Variance of RS | -0.50 | -0.41 | -0.02 |
| Variance Explained [%] | 35 | 24 | 15 |

**b**

| ***Middle*** | Factor 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Ramp Modulus | **0.95** | 0.04 | -0.15 | 0.11 |
| Equilibrium Modulus | **0.91** | 0.17 | 0.25 | -0.08 |
| Strength | **0.61** | 0.26 | 0.13 | 0.60 |
| COL Content | 0.14 | **0.85** | 0.22 | 0.00 |
| DNA Content | 0.10 | **0.77** | -0.32 | 0.13 |
| Fluorescence Ratio | 0.09 | **0.71** | 0.17 | 0.40 |
| GAG Content | -0.12 | -0.07 | **0.87** | 0.08 |
| Water Content | -0.34 | -0.19 | **-0.67** | -0.14 |
| Failure Strain | -0.25 | 0.07 | 0.29 | **0.86** |
| COL in aCT | -0.36 | -0.21 | 0.10 | **-0.63** |
| Variance Explained [%] | 25 | 20 | 16 | 17 |

**c**

| ***Deep*** | Factor 1 | 2 | 3 |
|---|---|---|---|
| Ramp Modulus | **0.95** | 0.07 | -0.06 |
| Strength | **0.82** | 0.38 | 0.15 |
| Equilibrium Modulus | **0.80** | -0.20 | 0.47 |
| Fluorescence Ratio | 0.03 | **0.80** | 0.10 |
| Failure Strain | -0.36 | **0.71** | 0.18 |
| COL in aCT | -0.15 | **-0.66** | 0.08 |
| COL Content | 0.18 | **0.59** | 0.50 |
| DNA Content | 0.30 | **0.57** | -0.11 |
| GAG Content | -0.04 | -0.10 | **0.84** |
| Water Content | -0.47 | -0.29 | **-0.72** |
| Variance Explained [%] | 27 | 25 | 18 |

**Table D.5:** Principal component analysis to identify dominant parameters in Aged NL, DGN, and OA samples of the lateral femoral condyle accounting for variations in parameters. Loading coefficients of variation and percent of total variance for each factor are shown for superficial (**a**), middle (**b**), and deep (**c**) layers, with dominant parameters in bold.

**a**

| ***Superficial*** | Factor 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Ramp Modulus | **0.94** | 0.00 | 0.11 | -0.07 | 0.05 |
| Equilibrium Modulus | **0.90** | -0.15 | -0.32 | 0.03 | 0.01 |
| Strength | **0.74** | -0.25 | -0.33 | -0.08 | -0.25 |
| DNA Content | 0.02 | **-0.83** | -0.19 | 0.03 | 0.03 |
| COL Content | 0.30 | **-0.75** | 0.17 | -0.15 | -0.06 |
| Fluorescence Ratio | 0.08 | **-0.66** | -0.23 | 0.44 | -0.16 |
| GAG Content | 0.11 | 0.13 | **-0.88** | 0.02 | 0.08 |
| Histopathology Index | -0.21 | 0.30 | **0.73** | 0.02 | 0.13 |
| Failure Strain | -0.14 | -0.24 | **-0.57** | -0.22 | -0.53 |
| Water Content | -0.48 | 0.13 | **0.56** | -0.14 | -0.18 |
| Variance of RS | -0.04 | 0.12 | 0.16 | **0.86** | 0.17 |
| COL in aCT | 0.06 | 0.36 | 0.23 | **-0.64** | 0.34 |
| Mean RS | 0.08 | 0.00 | 0.08 | 0.05 | **-0.91** |
| Variance Explained [%] | 20 | 16 | 18 | 11 | 11 |

**b**

| ***Middle*** | Factor 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Ramp Modulus | **0.93** | 0.08 | -0.20 | 0.08 |
| Strength | **0.86** | -0.03 | 0.41 | 0.19 |
| Equilibrium Modulus | **0.73** | -0.42 | -0.09 | 0.32 |
| GAG Content | -0.09 | **-0.92** | 0.14 | -0.01 |
| Water Content | -0.37 | **0.77** | -0.11 | -0.09 |
| Failure Strain | 0.00 | -0.09 | **0.87** | -0.11 |
| COL in aCT | 0.09 | 0.35 | **-0.53** | -0.33 |
| COL Content | 0.14 | -0.18 | -0.21 | **0.85** |
| DNA Content | 0.19 | 0.06 | 0.21 | **0.75** |
| Fluorescence Ratio | 0.27 | 0.46 | 0.47 | **0.52** |
| Variance Explained [%] | 24 | 20 | 16 | 18 |

**c**

| ***Deep*** | Factor 1 | 2 | 3 |
|---|---|---|---|
| Ramp Modulus | **0.93** | -0.06 | 0.08 |
| Equilibrium Modulus | **0.83** | 0.01 | -0.40 |
| DNA Content | **0.75** | 0.17 | 0.06 |
| Strength | **0.73** | 0.54 | 0.00 |
| COL Content | **0.65** | 0.30 | 0.30 |
| Failure Strain | -0.21 | **0.85** | -0.01 |
| Water Content | -0.33 | **-0.70** | 0.28 |
| COL in aCT | -0.27 | **-0.63** | 0.25 |
| Fluorescence Ratio | 0.43 | **0.57** | 0.41 |
| GAG Content | -0.05 | 0.26 | **-0.87** |
| Variance Explained [%] | 35 | 24 | 13 |

**Table D.6:** Principal component analysis to identify dominant parameters in Aged NL, DGN, and OA samples of the medial femoral condyle accounting for variations in parameters. Loading coefficients of variation and percent of total variance for each factor are shown for superficial (**a**), middle (**b**), and deep (**c**) layers, with dominant parameters in bold.

**a** **Lateral Femoral Condyle**

| ***Superficial*** | Strength | | Equilibrium Modulus | | Ramp Modulus | | Failure Strain | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Mean RS | 0.62 | **0.000** | 0.44 | **0.000** | 0.42 | **0.000** | 0.40 | **0.000** |
| Histopathology Index | 0.66 | **0.000** | 0.49 | **0.000** | 0.54 | **0.000** | 0.46 | **0.000** |
| Variance of RS | 0.28 | **0.002** | 0.23 | **0.006** | 0.12 | 0.052 | 0.18 | **0.017** |
| Water Content | 0.30 | **0.001** | 0.32 | **0.001** | 0.09 | 0.094 | 0.19 | **0.013** |
| GAG Content | 0.06 | 0.173 | 0.13 | **0.037** | 0.02 | 0.390 | 0.02 | 0.389 |
| DNA Content | 0.33 | **0.001** | 0.27 | **0.003** | 0.37 | **0.000** | 0.16 | **0.026** |
| COL Content | 0.20 | **0.009** | 0.25 | **0.003** | 0.10 | 0.066 | 0.00 | 0.967 |
| COL in aCT | 0.18 | **0.018** | 0.19 | **0.013** | 0.28 | **0.002** | 0.00 | 0.885 |
| Fluorescence Ratio | 0.48 | **0.000** | 0.34 | **0.001** | 0.33 | **0.001** | 0.45 | **0.000** |

**b**

| ***Middle*** | Strength | | Equilibrium Modulus | | Ramp Modulus | | Failure Strain | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Water Content | 0.20 | **0.011** | 0.18 | **0.017** | 0.03 | 0.327 | 0.06 | 0.167 |
| GAG Content | 0.00 | 0.859 | 0.02 | 0.445 | 0.04 | 0.290 | 0.10 | 0.072 |
| DNA Content | 0.12 | 0.058 | 0.03 | 0.334 | 0.03 | 0.385 | 0.02 | 0.490 |
| COL Content | 0.10 | 0.082 | 0.09 | 0.090 | 0.02 | 0.418 | 0.01 | 0.668 |
| COL in aCT | 0.20 | **0.013** | 0.07 | 0.139 | 0.18 | **0.018** | 0.09 | 0.108 |
| Fluorescence Ratio | 0.20 | **0.013** | 0.04 | 0.320 | 0.03 | 0.370 | 0.11 | 0.067 |

**c**

| ***Deep*** | Strength | | Equilibrium Modulus | | Ramp Modulus | | Failure Strain | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Water Content | 0.34 | **0.001** | 0.44 | **0.000** | 0.14 | **0.040** | 0.03 | 0.350 |
| GAG Content | 0.00 | 0.785 | 0.11 | 0.063 | 0.00 | 0.871 | 0.00 | 0.782 |
| DNA Content | 0.12 | 0.064 | 0.00 | 0.721 | 0.06 | 0.208 | 0.02 | 0.491 |
| COL Content | 0.14 | **0.037** | 0.06 | 0.195 | 0.01 | 0.523 | 0.07 | 0.139 |
| COL in aCT | 0.04 | 0.263 | 0.00 | 0.848 | 0.03 | 0.394 | 0.06 | 0.190 |
| Fluorescence Ratio | 0.11 | 0.081 | 0.00 | 0.748 | 0.02 | 0.438 | 0.31 | **0.002** |

**Table D.7:** Correlation coefficients of determination ($R^2$) and significance (p) values of univariate linear regression analysis for Aged NL, DGN, and OA samples of the lateral femoral condyle. Values are shown for superficial (**a**), middle (**b**), and deep (**c**) layers, with dominant parameters in bold.

**a** **Medial Femoral Condyle**

| ***Superficial*** | Strength | | Equilibrium Modulus | | Ramp Modulus | | Failure Strain | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Mean RS | 0.01 | 0.531 | 0.00 | 0.833 | 0.00 | 0.978 | 0.07 | 0.133 |
| Histopathology Index | 0.23 | **0.007** | 0.21 | **0.011** | 0.02 | 0.510 | 0.20 | **0.013** |
| Variance of RS | 0.02 | 0.448 | 0.01 | 0.695 | 0.01 | 0.632 | 0.06 | 0.168 |
| Water Content | 0.13 | **0.047** | 0.43 | **0.000** | 0.09 | 0.108 | 0.01 | 0.627 |
| GAG Content | 0.07 | 0.159 | 0.11 | 0.060 | 0.00 | 0.806 | 0.11 | 0.058 |
| DNA Content | 0.09 | 0.112 | 0.05 | 0.257 | 0.00 | 0.943 | 0.06 | 0.196 |
| COL Content | 0.09 | 0.104 | 0.11 | 0.068 | 0.07 | 0.153 | 0.01 | 0.595 |
| COL in aCT | 0.02 | 0.444 | 0.01 | 0.630 | 0.02 | 0.468 | 0.04 | 0.289 |
| Fluorescence Ratio | 0.09 | 0.101 | 0.05 | 0.233 | 0.00 | 0.771 | 0.08 | 0.126 |

**b**

| ***Middle*** | Strength | | Equilibrium Modulus | | Ramp Modulus | | Failure Strain | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Water Content | 0.132 | **0.049** | 0.234 | **0.007** | 0.068 | 0.165 | 0.009 | 0.615 |
| GAG Content | 0.001 | 0.887 | 0.077 | 0.123 | 0.032 | 0.326 | 0.048 | 0.229 |
| DNA Content | 0.154 | **0.026** | 0.090 | 0.095 | 0.043 | 0.253 | 0.020 | 0.438 |
| COL Content | 0.048 | 0.228 | 0.224 | **0.006** | 0.041 | 0.266 | 0.016 | 0.484 |
| COL in aCT | 0.037 | 0.292 | 0.028 | 0.364 | 0.009 | 0.61 | 0.055 | 0.198 |
| Fluorescence Ratio | 0.245 | **0.004** | 0.006 | 0.678 | 0.047 | 0.233 | 0.039 | 0.279 |

**c**

| ***Deep*** | Strength | | Equilibrium Modulus | | Ramp Modulus | | Failure Strain | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** | **$R^2$** | **p** |
| Water Content | 0.199 | **0.017** | 0.102 | 0.098 | 0.038 | 0.326 | 0.169 | **0.03** |
| GAG Content | 0.020 | 0.466 | 0.066 | 0.181 | 0.023 | 0.438 | 0.044 | 0.275 |
| DNA Content | 0.238 | **0.007** | 0.261 | **0.005** | 0.321 | **0.002** | 0.001 | 0.846 |
| COL Content | 0.348 | **0.001** | 0.070 | 0.166 | 0.231 | **0.01** | 0.000 | 0.975 |
| COL in aCT | 0.203 | **0.014** | 0.086 | 0.123 | 0.047 | 0.268 | 0.107 | 0.083 |
| Fluorescence Ratio | 0.399 | **0.002** | 0.048 | 0.342 | 0.090 | 0.199 | 0.220 | **0.032** |

**Table D.8:** Correlation coefficients of determination ($R^2$) and significance (p) values of univariate linear regression analysis for Aged NL, DGN, and OA samples of the medial femoral condyle. Values are shown for superficial (**a**), middle (**b**), and deep (**c**) layers, with dominant parameters in bold.

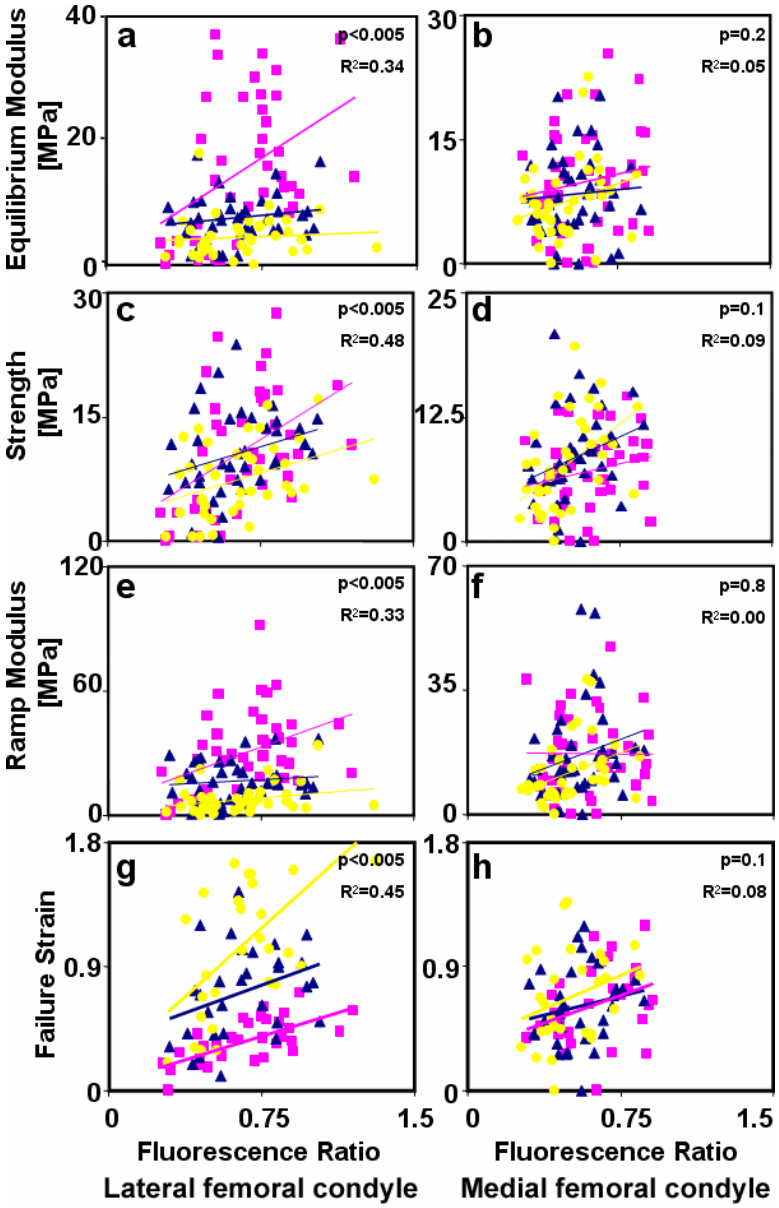



**Figure D.6:** Relationships between tensile properties and fluorescence ratio of Aged NL, DGN, and OA samples. Relationships between the tensile equilibrium modulus (**a**, **b**), strength (**c**, **d**), ramp modulus (**e**, **f**), and failure strain (**g**, **h**) and fluorescence ratio for data from superficial (■), middle (▲), and deep (●) layers of the lateral (**a**, **c**, **e**, **g**) and medial (**b**, **d**, **f**, **h**) femoral condyles are shown. $R^2$ and p-values shown are for the relationship with data of the superficial layer. Lines represent linear regression fits and are meant only to show trends.

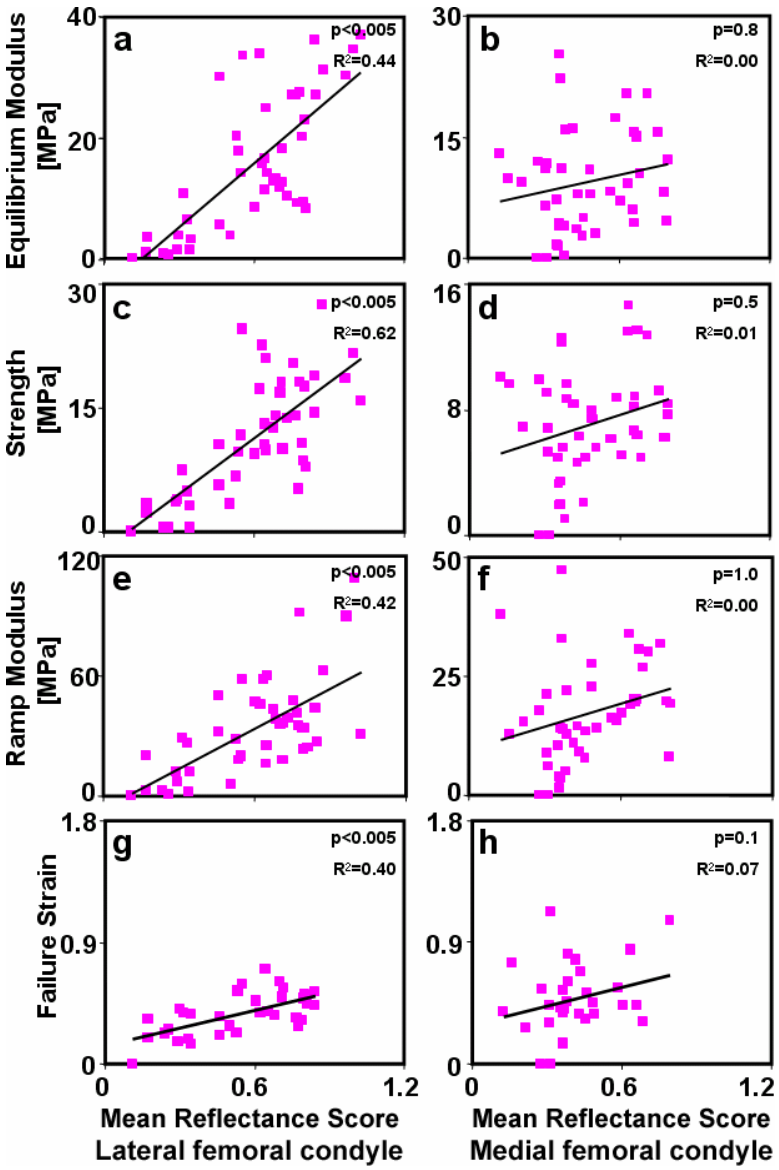



**Figure D.7:** Relationships between tensile properties and mean reflectance score of Aged NL, DGN, and OA samples are plotted as described in Figure D.6.

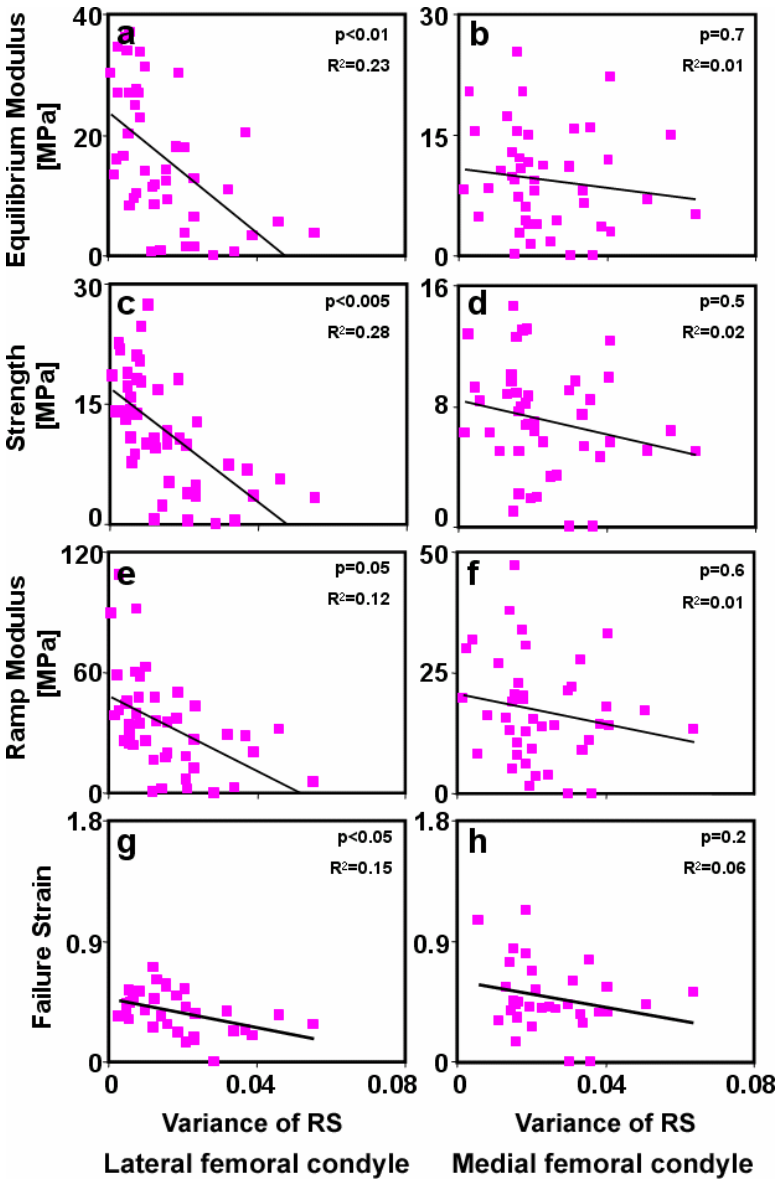



**Figure D.8:** Relationships between tensile properties and variance of the reflectance score (RS) of Aged NL, DGN, and OA samples are plotted as described in Figure D.6.

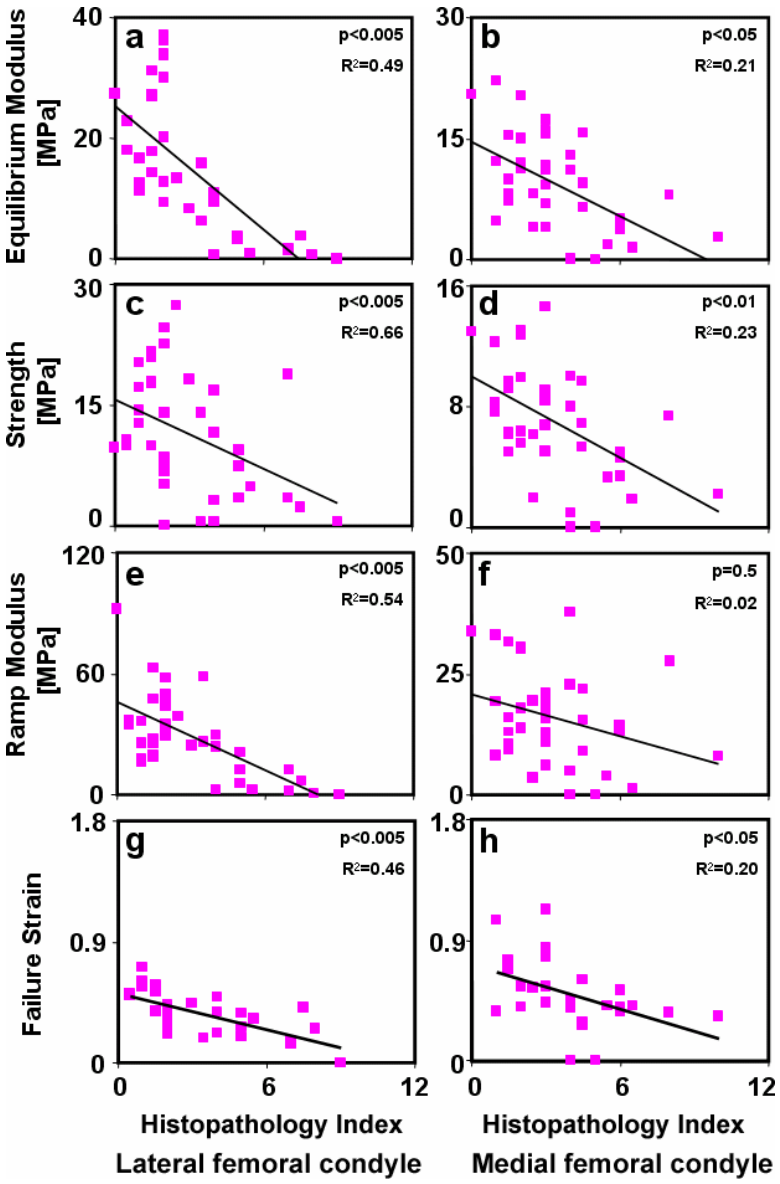



**Figure D.9:** Relationships between tensile properties and histopathology index of Aged NL, DGN, and OA samples are plotted as described in Figure D.6.

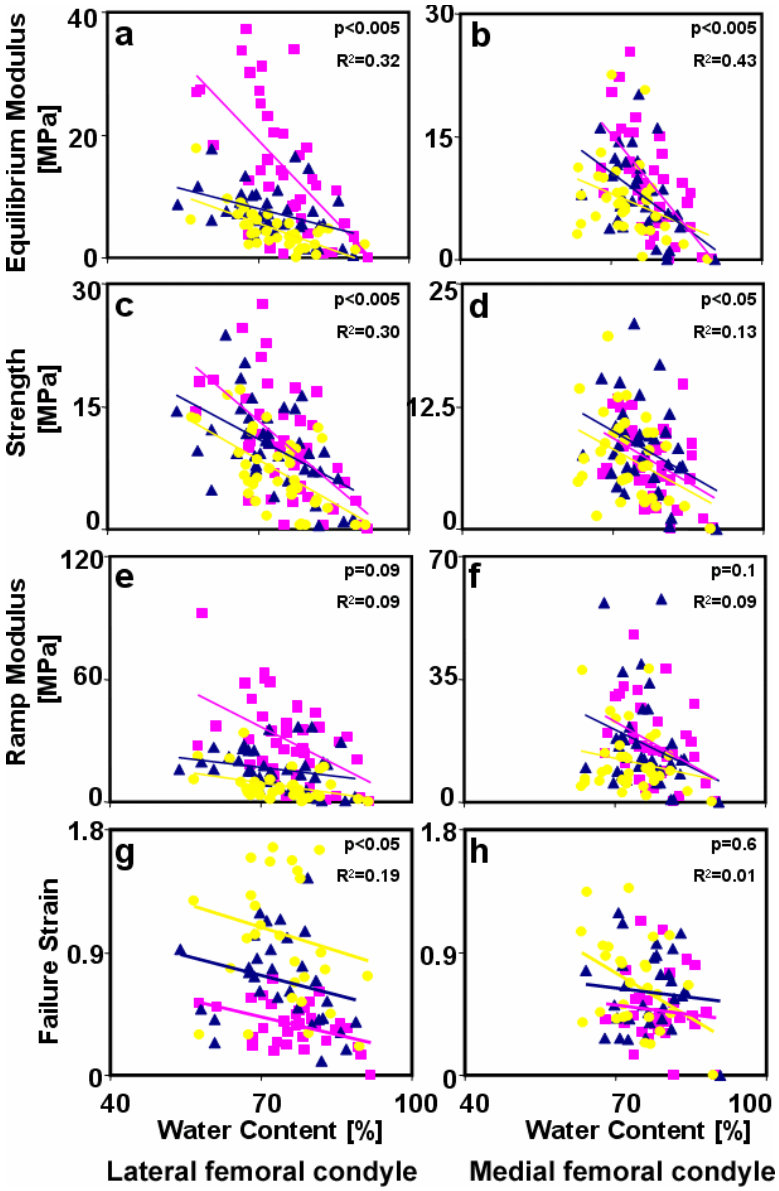



**Figure D.10:** Relationships between tensile properties and water content of Aged NL, DGN, and OA samples are plotted as described in Figure D.6.

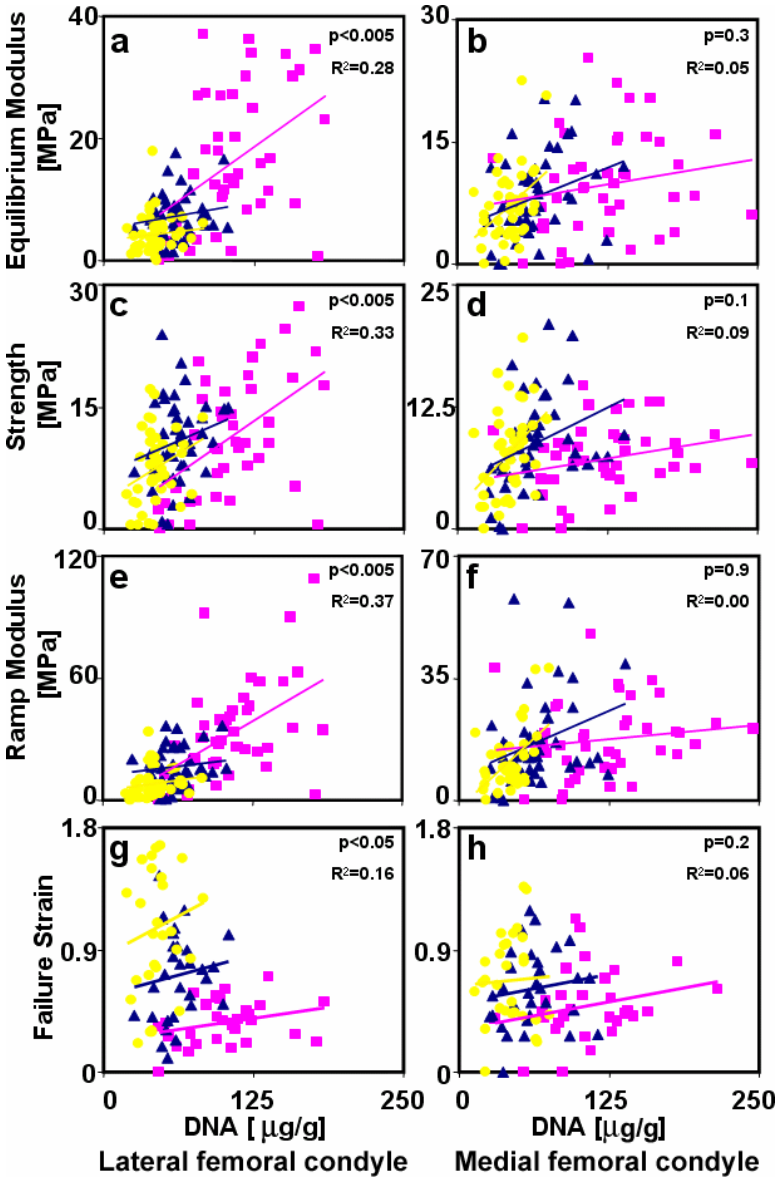



**Figure D.11:** Relationships between tensile properties and DNA content of Aged NL, DGN, and OA samples are plotted as described in Figure D.6.

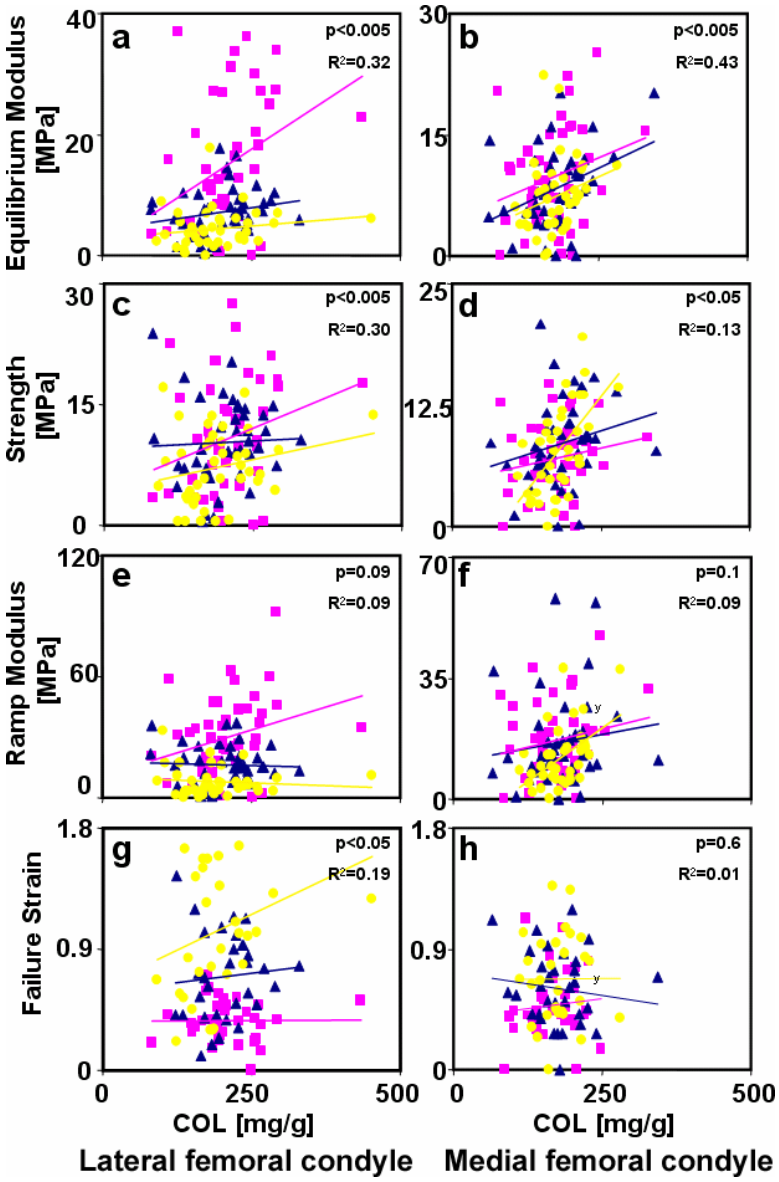



**Figure D.12:** Relationships between tensile properties and COL content of Aged NL, DGN, and OA samples are plotted as described in Figure D.6.

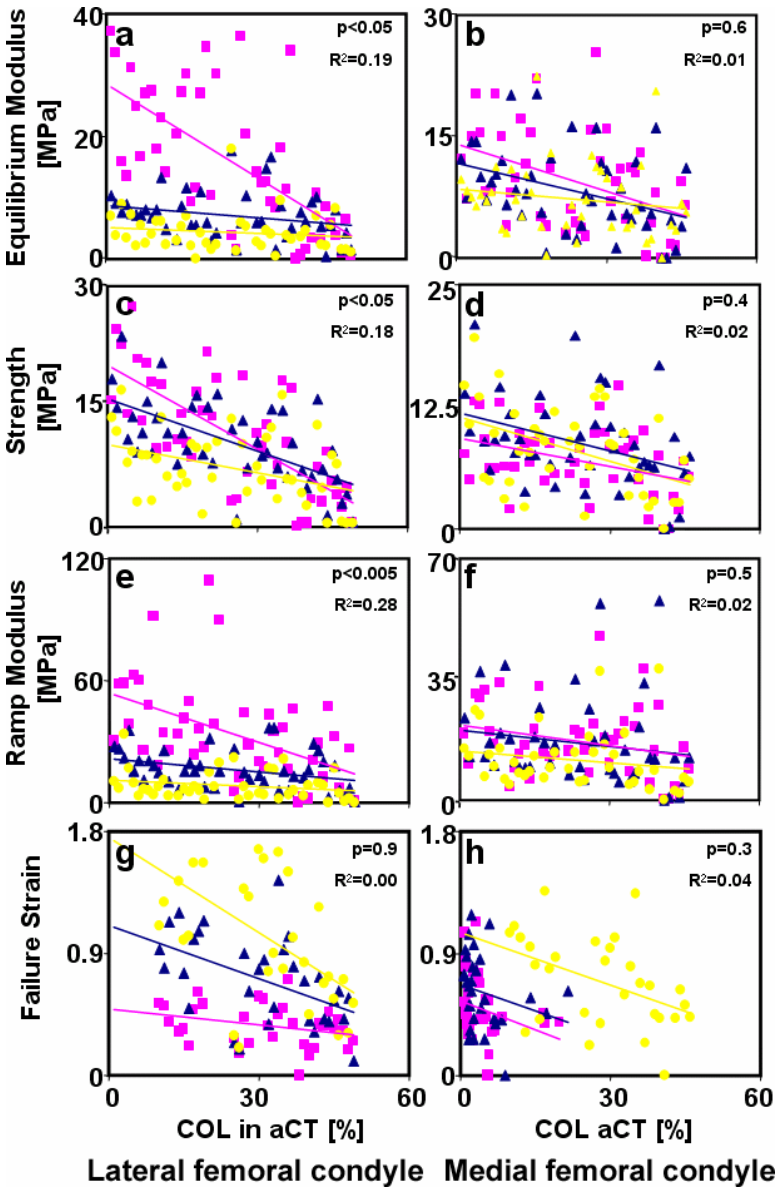



**Figure D.13:** Relationships between tensile properties and degraded collagen (COL in aCT) content of Aged NL, DGN, and OA samples are plotted as described in Figure D.6.

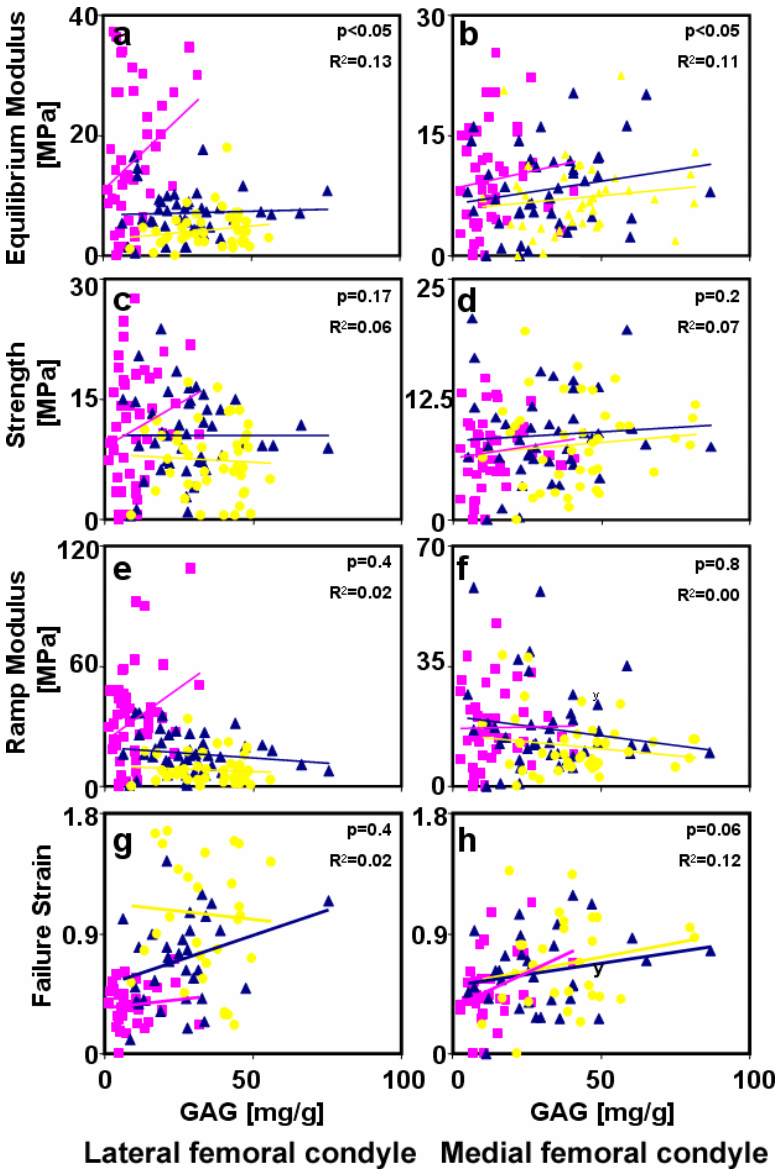



**Figure D.14:** Relationships between tensile properties and glycosaminoglycan (GAG) content of Aged NL, DGN, and OA samples are plotted as described in Figure D.6.

## D.4 Discussion

While much more information can be gleaned from these analyses, some important relationships were highlighted. These relationships were site- and depth-dependent, being most evident in the superficial layer of the LFC and the deep layer of the MFC. In agreement with results of Chapter 2, for Young NL, Middle NL, and Old NL samples, tensile properties decreased with increasing age, decreased with a decrease in the mean reflectance score, and decreased with an increase in the variance of the reflectance score in the superficial layer of the LFC. Other structural and biochemical properties were not strongly related to tensile weakening of the superficial layer. More work is needed to fully elucidate the causes and consequences of age-associated tensile weakening. The results of this study point to a role for surface wear and fatigue as major changes in early age-associated cartilage deterioration. The extent and localization of changes in particular tissue properties set the stage for further studies with specific and graded manipulations to test the mechanistic relationship for the sequence of events described in Figure 2.4.

In agreement with results of Chapter 3, for age-matched NL, DGN, and OA samples, the parameters most strongly related to tensile weakening in the superficial layer of the LFC were the fluorescence ratio, the mean reflectance score, and the histopathology score. The tensile integrity decreased with decreasing fluorescence ratio, decreased with decreasing mean reflectance score, and decreased with increasing histopathology index in the superficial layer of the LFC. This study points to disruption of the articular surface and an altered collagen network as contributing factors in the progression of cartilage weakening in early degeneration (Figure 3.4).

Additional work is needed to elucidate the mechanisms behind surface wear and collagen network remodeling in early cartilage degeneration and the sequence of events leading to overt cartilage fibrillation and osteoarthritis.