# ASSESSMENT OF BIOMARKER PRODUCTION BY OSTEOARTHRITIC OSTEOCHONDRAL TISSUES AND CORRELATION TO THE BIOMECHANICAL, BIOCHEMICAL, AND HISTOLOGICAL PROPERTIES

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

ASSESSMENT OF BIOMARKER PRODUCTION BY OSTEOARTHRITIC OSTEOCHONDRAL TISSUES AND CORRELATION TO THE BIOMECHANICAL, BIOCHEMICAL, AND HISTOLOGICAL PROPERTIES

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and hereby certify that, in their opinion, it is worthy of acceptance.

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# ASSESSMENT OF BIOMARKER PRODUCTION BY OSTEOARTHRITIC OSTEOCHONDRAL TISSUES AND CORRELATION TO THE BIOMECHANICAL, BIOCHEMICAL, AND HISTOLOGICAL PROPERTIES

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## ABSTRACT

Osteoarthritis (OA) is the most prevalent form of arthritis and is a significant cause of pain and disability worldwide. Treatment options available are only capable of controlling symptoms until progression to end-stage OA and qualifications for total knee replacement are met. To this regard, there is a strong need for the development of diagnostic tools and therapeutic interventions targeting earlier stages before irreversible damage occurs. It is generally accepted that changes in the structural orientation, biochemical parameters, and biomechanical properties together with inflammation are the main contributors to the development and progression of OA. Early stages of OA are characterized by alterations in the microscopic structure and microenvironment of the cartilage progressing to irreversible macroscopic tissue damage. Paralleling and in response to these changes, chondrocytes become activated, increasing the production of proteins involved in the degradation and synthesis of extracellular matrix (ECM) components and those involved in inflammation, which could serve as relevant biomarkers.

In the past decade, much attention in OA research has focused on the use of biomarkers for the identification and targeting of earlier stages of OA. In this body of research, we aimed to investigate relationships between the production of biomarkers and changes in biochemical, biomechanical, and structural properties of osteochondral tissue from patients undergoing total knee arthroplasty. Following 3 or 4 days of culture, osteochondral explants were tested for tissue puncture force (TPF) using a needle puncture test, aggregate modulus ($H_A$) and permeability ($\kappa$) using unconfined compression testing, and histology using the Osteoarthritis Research Society International (OARSI) scoring system, or chondrocyte viability using fluorescent live/dead stain imaging, and tissue GAG and HP content. Culture media were analyzed for the production of a multitude of biomarkers including cytokines, matrix metalloproteinases (MMPs), inflammatory molecules, apoptosis mediators, and death receptors.

We have identified interrelationships between chondrocyte viability, surface integrity measured by TPF, compressive and hydrostatic biomechanical properties, histopathology scores, and ECM content of OA cartilage tissue. Specifically, we show positive relationships between viable cell density, aggregate modulus, and TPF, and negative relationships between total histology score and viable cell density or aggregate modulus. Total histology score and TPF were associated with changes in collagen and proteoglycan content. Furthermore, we show that viable cell density, aggregate modulus, and TPF all had positive relationships with cytokine production and negative relationships with MMP production.

The production of biomarkers was also interrelated. Interestingly, there was a negative relationship between cytokine and MMP production, which was consistent throughout the study as seen in the opposing relationships with tissue properties. Cytokines and MMPs showed opposing relationships with NO, $PGE_2$, and death receptors. We also found that cytokines GRO-α, IL-6, IL-8, and MCP-1 tended to correlate as a group. Similarly, MMP-1, MMP-9, MMP-13, and in a negative manner MMP-2 tended to correlate as a group. However, we did not find this to be true for NO and $PGE_2$ which did not have a significant correlation to each other.

We concluded that biomarkers of inflammation, degradation, apoptosis, and chondrocyte viability produced by osteoarthritic cartilage from various regions of the osteoarthritic joint show moderate to strong correlations to each other suggesting that there is a correlation to OA biomarker production and the severity of pathology. Furthermore, these biomarkers showed correlations to changes in tissue biochemical, biomechanical, and structural properties. Thus, panels assessing levels of these biomarkers in bodily fluids such as synovial fluid, serum, and/ or urine have the potential to discriminate presence and severity of knee joint health, and possibly subgroups of OA, in the clinical setting.

# CHAPTER 1: Literature Review

## Properties of Articular Cartilage

Articular cartilage is an avascular connective tissue with a low coefficient of friction that covers the surfaces of diarthrodial joints. The main function of the cartilage is to provide a smooth, lubricating, and load-bearing surface protecting the joint from mechanical stressors and sustaining normal movement of the joint [1-5]. In the joint, cartilage is subject to a variety of mechanical forces, namely compressive, shear, and hydrostatic, as well as osmotic forces. These stressors are created during joint loading and movement. Tissue compression results the two articulating joint surfaces pressing against each other during weight bearing and muscle-driven loading. Shear stress is applied to the cartilage tissue at the two joint surfaces move along each other during joint movement. Hydrostatic pressure occurs in the cartilage tissue because proteoglycans within the tissue attracts synovial fluid into the tissue, which resists the compressive forces applied to the tissue. Osmotic pressure within the cartilage tissue results from the changes in synovial fluid solute concentration and fluctuations of fluid within the extracellular matrix (ECM) during loading [3]. The unique structure, organization, and properties of cartilage ECM function to diffuse these forces under physiological conditions [2-9].

The extracellular matrix of cartilage is mainly composed of collagen, proteoglycans, and water. The main type of collagen in cartilage is collagen type II which is organized into intrinsic networks [10]. This network of collagen fibers

provide cartilage with tensile stiffness [1, 3, 10, 11]**.** Proteoglycans and water reside within the interfibrillar spaces of the collagen network. The main proteoglycan in cartilage is aggrecan, which binds to hyaluronic acid allowing for the interaction between collagen and proteoglycans [11]. Proteoglycans are highly negatively charged and therefore attract water molecules into the matrix. This attraction of water molecules into the matrix creates a swelling pressure which constitutes the compressive property of cartilage [2, 5, 10-12]. Together, the interactions within the ECM modulate the mechanical stresses, strains, and fluid flow in the articular cartilage [1, 2, 4, 5, 13]. Numerous studies have reported a positive relationship between the intrinsic compressive and tensile properties with the biochemical content of normal articular cartilage [14-16].

The content and structural organization of the ECM components vary through the depth of the tissue and also relative to chondrocyte phenotype [1, 2, 4, 12, 14, 15]. These compositional and structural differences lead to differing mechanical properties throughout the tissue. Cartilage is divided into the superficial, middle, deep, and calcified zone from the surface of the cartilage down to the subchondral bone, respectively. In the superficial zone, collagen fibers are densely packed and orientated parallel to the surface providing the high tensile strength of this zone [1, 10, 11, 17]. There is also a low concentration of proteoglycans in the superficial layer [5, 10]. The most superficial layer, called the lamina splendens, is composed of a finer meshwork of fibers which serves as a membrane that transfers loads from directly loaded regions to adjacent regions and protecting the underlying cartilage from forming cracks under physiological

mechanical stresses **[1, 5, 8, 12]**. Lubricin molecules secreted into the joint coat the lamina spendens further providing boundary lubrication and preventing cell and protein adhesion [18]. The proteoglycan content increases in concentration with the depth of the tissue providing the tissue with its compressive resistance [5, 10] In the middle layer, the collagen fibers are less densely packed with a less organized orientation and become oriented perpendicular to the subchondral bone in the deep zone [2, 10] The compressive, elastic, and equilibrium properties increase with depth, which can be explained in part by the increased concentration of proteoglycans from the superficial layer to the deep layer [2, 5, 12, 17]. In contrast, collagen fibers become less densely packed with depth and therefore, the tensile stiffness decreases from the superficial to deep zone [1, 10, 11]**.** Nonetheless, together, the varying composition, structural organization, and interactions within the ECM components constitute the biomechanical properties of the tissue.

Chondrocytes are the primary cells in cartilage and therefore are the primary source of metabolic turnover. Chondrocytes are embedded within the ECM and like the ECM, their distribution varies throughout the cartilage. In normal cartilage, chondrocytes are relatively quiescent providing a low matrix turnover rate [2, 3, 9]. Furthermore, mechanical and chemical receptors expressed by chondrocytes maintain homeostasis during physiological loading by signaling the production of catabolic and anabolic molecules [1, 2, 7, 19, 20]. The balanced synthesis and degradation of the extracellular matrix has been shown to be essential for the normal function of articular cartilage. Disruption in

this metabolic turnover, by mechanical or chemical stresses, can initiate a cascade of cartilage degradative processes [2, 13, 19, 21]. Furthermore, once degradation of cartilage is initiated, the tissue progresses into downward cycles of irreversible cartilage destruction.

**Osteoarthritis and Associated Changes in Cartilage**

Osteoarthritis is the most common degradative joint disease and is a significant cause of pain and disability worldwide [22]. The joint is now commonly recognized as an organ comprised of the many tissues within the joint, and all of the tissues within the joint have the potential to be effected by and contribute to the development of OA clinically. In addition to changes in articular cartilage, subchondral bone, synovial tissue, infrapatellar fat pad (IPFP), the meniscus, ligaments, and tendons all play a role in the disease cascade [8]. However, cartilage degradation is the hallmark for all forms of OA, and once initiated, it is irreversible [8]. Cartilage degradation is a multifactorial process, characterized by the disruption of chondrocyte metabolism, loss of extracellular matrix components, and alterations in the biomechanical properties and structural organization of the tissue [1, 2, 4-7, 12, 19, 20].

Age, gender, traumatic injury, obesity, and genetics are risk factors associated with initiation and progression of OA [23]. It has also been hypothesized that these risk factors can represent different subgroups of OA such as post-traumatic OA (PTOA), age related OA, and obesity related OA [8]. Regardless of whether traumatic injury, prolonged mechanical wear, or inflammation initiates the progressive events of OA, all types eventually lead to

the macroscopic degeneration and loss of cartilage tissue resulting in bone on bone loading associated with end-stages of the disease. However, early stages of OA result in microscopic alterations of the structural and biochemical properties of the cartilage [2]. Many studies report the importance of changes in the microenvironment of the cartilage tissue resulting in the up-regulation of both matrix component synthesis as well as matrix degrading enzymes, triggering further destruction of the matrix and ultimately leading to increased calcification, chondrocyte hypertrophy, clonal clustering, and chondrocyte death [1, 2, 4, 5, 16, 24]. Furthermore, these degradative events lead to changes in the biomechanical properties of the tissue, enhancing the tissues susceptibility to mechanical damage.

Abnormal mechanical loading of the joint is reported to occur not only during traumatic injury, but also with altered weight bearing and gait patterns in obese patients, and in patients with joint pain associated with inflammation [9, 23, 25]. Therefore, changes in mechanical forces on the joint play a key role in the initiation and progression of OA. Supraphysiological loading, or impact loads, induce focal lesions, degradation, and mechanical signal transduction, initiating and/ or exacerbating the progression of OA [3, 8, 12]. Supraphysiological compression and high fluid shear stress of normal chondrocytes stimulate increased signal transduction and expression of cytokines, inflammatory molecules, and degradative enzymes *in vitro* [26-28]. Disorganization and damage to superficial collagen networks, increased histological grade, as well as upregulation of degradative enzymes are observed in response to injury induced

by experimental PTOA animal models [29-31]. Furthermore these changes are observed in cartilage from patients undergoing ACL reconstruction [32].

In addition to supraphysiological loads, alterations in diffusion or dissipation of normal loads are also associated with degenerative changes [9]. These altered distributions of normal loads can be a result of altered gait and weight bearing properties associated with OA risk factors, or focal damage to the cartilage within the joint. Thinning and loss of the superficial zone increases the magnitude of load experienced by the less tensile resistant middle and deep zones [10]. To compensate for these changes, cartilage undergoes compositional and structural reorganization which results in a change in cartilage tissue biomechanical properties [12, 17, 30, 33].

Microscopic and macroscopic degenerative changes of cartilage typically first appear in the superficial zone [8, 10, 11]. Disruption in the collagen network, decreased concentration of proteoglycans, and decreased thickness of the superficial layer are associated with early microscopic changes in OA [17, 30, 34]. This initial tissue damage is followed by changes in collagen organization and proteoglycan content of the middle and deep zones, resulting in increased swelling and tissue thickness [1, 10]. Increased calcification and tidemark advancement have also been associated with early changes in OA [35]. These early changes in the middle and deep zones of the cartilage tissue are hypothesized to be a compensatory effort in response to tensile failure of the superficial layer resulting in a change in the transmission of mechanical loads to

these layers of the tissue [10, 30, 33]. Ultimately these changes lead to macroscopic fibrillation and loss of cartilage structure and function.

As the ECM composition and structure throughout the layers of cartilage are altered, the compressive, elastic, equilibrium, and tensile properties of the tissue change in an attempt to compensate for the abnormal loads being applied to the tissue [5, 10, 12]. In general, compressive, elastic, and tensile properties are decreased, and equilibrium and overall stiffness is increased during the progression of OA [4, 5, 9, 10]. In early stages, as the collagen meshwork becomes disorganized and proteoglycans are lost, elastic modulus becomes less uniform throughout the tissue, collagen fibrils become stiffened in the superficial and calcified zone, and the cartilage thickens as a result of increased swelling due to the loss of collagen network integrity within the tissue [17, 30, 33]. In OA cartilage from thumb carpometacarpal joints, the compressive modulus was decreased and collagen stiffness was increased [36]. Furthermore, the stiffening of collagen together with a loss in proteoglycan content resulted in an increase in water content.

Chondrocytes interact with the ECM and surrounding microenvironment through mechanical and chemical membrane receptors [3]. Therefore, changes in composition, structural organization, and biomechanical properties of the ECM, as well as abnormal loading leads to the activation of normally quiescent chondrocytes and disrupts metabolic homeostasis. These changes to the tissue interact with chondrocyte membrane receptors to both stimulate and inhibit signal transduction pathways associated with ECM synthesis and repair, degradative

enzyme production, inflammation and chondrocyte death [9, 37-39]. Therefore, because OA typically develops as a focal disease affecting specific areas of the cartilage surface, changes in biomarker production are varied across the surface of the joint, potentially paralleling changes in biochemical, biomechanical, and structural organization of OA cartilage. As a result, it is possible that identifying biomarker production patterns associated with the tissues physical properties may provide insight into the pathogenesis and pathology of OA clinically.

**Biomarkers in Osteoarthritis**

Currently, the diagnosis of OA is based on clinical assessments, diagnostic imaging findings, and macroscopic (arthroscopic, gross) changes in the joint. However these changes are usually associated with late-stage disease when damage is irreversible. Furthermore, most of the available treatment options are limited to palliative therapies aimed to reduce pain until surgical joint replacement. The FDA has recognized these limitations and the need for development of diagnostic tools and therapeutic interventions targeting earlier stages of disease that precede irreversible degradation [40]. Therefore, much attention in the past decade has been directed towards the use of biomarkers that are associated with early biomechanical, biochemical, and structural changes of the disease [41-43].

Biomarkers for OA can be classified by (B) burden of disease, (I) investigative, (P) prognostic, (E) efficacy of intervention, and (D) diagnostic (BIPED), a system developed by the OA Biomarker Network in an attempt to enhance biomarker validation and analysis [44]. Simplified, B markers asses

severity of extent, I markers are those in which further research is needed to establish the relationship to various parameters of ECM turnover, P markers predict future onset or progression of OA, E markers asses the efficacy of treatment among persons with the disease, and D markers can distinguish between individuals with or without the disease. Since the development of this classification system, numerous biomarkers have been identified and investigated (for review see [43]). Among these, we are interested in pro-inflammatory cytokines, matrix metalloproteinases (MMPs), apoptosis mediators, and inflammatory molecules nitric oxide (NO) and prostaglandin $E_2$ ($PGE_2$).

In addition to being increased in the serum and synovial fluid of patients with OA [43, 45, 46], the expression and concentration of these biomarkers are up regulated in experimentally induced *in vitro* and *in vivo* models of OA [2, 3, 8, 9, 38, 47-49]. Supraphysiological and continuous fluid shear stress increases the production of IL-1β, IL-6, and MMP-9 [28, 50] and supraphysiological dynamic or intermittent compression increases IL-1β, TNF-α, MMP-2, MMP-9, MMP-13, $PGE_2$, and NO *in vitro* [26, 27, 51]. *In vivo* studies found increased NO production by cartilage from anterior cruciate ligament transected (ACLT) knees of rabbits and intraarticular injection of caspase inhibitors reduces the severity of lesions in rabbit ACLT knees [31, 52].

Furthermore, these biomarkers exacerbate metabolic disruption by further activation of signal transduction pathways and contributing to the increased degradation and decreased synthesis of the ECM [2, 9, 38]. Specifically, MMPs are degradative enzymes, and MMP-1, MMP-2, MMP-9, and MMP-13 target the

degradation of collagen and proteoglycans [11, 53]. Proinflammatory cytokines, such as IL-1β and TNF-α, are shown to suppress the expression of ECM synthesis genes and thus decrease synthesis of collagen and proteoglycans [4, 7, 42, 54]. NO and $PGE_2$ are associated with inhibition of ECM synthesis, chondrocyte hypertrophy, increased calcification, and apoptosis [54-57]. Finally, stimulation of chondrocytes and cartilage explants with IL-1β, TNF-α, IL-6, GRO-α, IL-8, or MCP-1 increases the production of MMPs, $PGE_2$, and NO [58-63].

During the past decade, many significant advances have been made aiding in the understanding of OA. Recently, strategies have been aimed towards studying the relationships between the biomechanical, structural, and biochemical properties of osteoarthritic cartilage, and the roles these properties play in the progression of the disease [2, 4, 5, 33, 64, 65]. Additionally, many studies have investigated the metabolism of the cartilage tissue and the roles of specific biomarkers in the pathology of the disease through direct or indirect analysis [4, 6, 7, 13, 19-21, 23, 66]. However, to the author's knowledge, the relationship between the production of specific biomarkers by the cartilage tissue and the changes in biomechanical, biochemical, and structural properties of the tissue has not been investigated thoroughly. Traditional approaches have focused on characterizing the effects of one or few biomarkers on specific events in OA pathway [54]. Therefore, the purposes of this study are to analyze the metabolism of osteochondral tissue obtained from patients undergoing total knee arthroplasty and correlate the production of a variety of known OA biomarkers to the biochemical, biomechanical, and structural properties of the tissue.

## CHAPTER 2: Introduction

Osteoarthritis (OA) is a progressive degenerative disease that affects approximately 22% of adults and is associated with total costs of more than $100 billion annually [22]. Currently, there are no treatment options available that can prevent the progression of this disease or restore normal integrity and function of the articular cartilage. OA is a multifactorial disease driven by inflammation, disruption in the biochemical composition, alterations in the structural organization, and loss of biomechanical properties of articular cartilage. Specific changes in these physical and metabolic properties of osteoarthritic and osteoarthritic-like tissues are well documented [2, 4, 7, 15, 19]. However, how these pathologic changes to the cartilage tissue interrelate to drive the pathology of OA is not well understood.

It is now understood that OA is a disease of the entire joint organ and not just articular cartilage. In addition to cartilage loss, changes in the subchondral bone, synovium, infrapatellar fat pad (IPFP), ligaments, and tendons all contribute to whole-joint disease processes and resultant pain and dysfunction [8]. Numerous studies indicate that changes in the microenvironment are important driving forces behind the disruption of cartilage structure and function [67]. The data from these studies suggest significant correlations between biomechanical properties and extracellular matrix (ECM) composition, and organization [33, 36, 65, 68].

Furthermore, changes in the cartilage tissues metabolism during the pathogenesis and progression of OA have been extensively researched, and many in vivo and in vitro studies have found changes in the production of biomarkers indicative of inflammation, apoptosis, and degradation [2, 7, 15, 21]. However, to the author's knowledge, the relationships between the production of biomarkers by OA tissue and the biochemical, biomechanical, and structural properties of articular cartilage have not been fully elucidated.

Therefore, this study was designed to analyze the in vitro biomarker production of osteochondral tissues collected from the knees of patients undergoing total knee arthroplasty, and correlate the production of these biomarkers to the tissues biomechanical, histological, and biochemical properties. We hypothesized that 1) there will be strong ($r>0.7$) correlations among relevant biomarkers produced by the cartilage tissue during culture; and 2) strong ($r>0.7$) correlations will be found between the physical properties and the biomarker synthesis of the tissue.

## CHAPTER 3: Materials and Methods

### Reagents and Instruments:

An 8mm OATS harvester was purchased from Arthrex Inc., Naples, Florida, USA. Dulbecco's Modified Essential Medium (DMEM), and Perchloric acid were purchased from Thermo Fisher Scientific Inc. Serum replacement solution was purchased from Becton Dickinson, Franklin Lakes, NJ, USA. Human Cytokine/Chemokine Magnetic Bead Panel was purchased from Millipore, Billerica, MA, USA. Human MMP Magnetic Luminex Performance Assay was purchased from R&D System, Minneapolis, Minnesota, USA. PGE2 Express EIA Kit was purchased from Cayman Chemical, Ann Arbor, MI, USA. Collagen Type II Cleavage (C2C) ELISA and Aggrecan Chondroitan Sulfate 846 Epitope (CS846) ELISA were purchased from IBEX Pharmaceuticals Inc., Montreal, Quebec, Canada. Calcein AM fluorescent stain, Human Death Receptor 3-Plex Panel and Human Apoptosis 3-Plex Panel were purchased from Invitrogen Carlsbad, CA, USA. SensoLyte® 520 Generic MMP Activity Kit was purchased from Anaspec Inc., Fremont, CA, USA. Sodium phosphate, EDTA, Dithiothreitol, Papain, 1,9-Dimethyl-Methylene Blue (DMMB), Glycine, chondroitin sulfate A, Chloramine-T, L-hydroxyproline, and n-Propanol were purchased from Sigma-Aldrich, St. Louis, MO, USA. Griess Reagent System was purchased from Promega Corp., Madison, WI, USA.

**Patients:**

Osteochondral tissue typically discarded during total joint arthroplasty was collected from 7 knee-joints of 6 patients (4 male and 2 female) undergoing total knee arthroplasty for severe OA. A breakdown of patient's demographics is outlined in Table 3-1. All surgical procedures were indicated by clinical (pain, loss of function and mobility) and radiological signs of OA. Informed consent was obtained from all patients after the nature of all examinations had been fully explained. All procedures were performed with IRB approval (IRB# 1208392).

**Sample Preparation and Culture:**

At joint replacement surgery, remaining osteochondral tissue was obtained from the femoral condyle (n=7) and tibial plateau (n=3), and kept in phosphate buffered saline for less than 4 hours. Osteochondral explants (n=139) including subchondral bone were created from these tissues using an OATS harvester (8mm). Explants were harvested from all regions of the joint where cartilage was still present. Tissue explants were cultured in 24 well tissue culture plates containing 2mls of DMEM supplemented with 1 U/mL penicillin, 100 µg/mL streptomycin, 2 µg/mL amphotericin B, 2mM L-glutamine, 50 µg/mL L-ascorbic acid, 0.1mM modified Eagle medium nonessential amino acids, 1mM sodium pyruvate, and 1X serum replacement solution containing insulin, transferrin, and selenous acid. Explants were cultured for 3-4 days at 37°C and 5%CO2. At the end of culture, media were collected for biomarker analysis and replenished for biomechanical testing.

**Biomechanical Testing:**

All explants were tested for the biomechanical properties using an Instron 8821S servo-hydraulic testing machine. Osteochondral explants were placed in a stainless steel well (8mm in diameter) and oriented so that the center of the cartilage surface was perpendicular to the ram. Cartilage thickness and tissue puncture force (TPF; N) was determined using a blunted 22g needle attached to the ram. Briefly, the blunted needle was advanced at 0.1mm/s while the force was monitored. An initial increase in force was noted as the needle contacted the cartilage surface. A sharp drop in the force was noted as the "membrane" was punctured. Finally, an increase in the force was noted when the subchondral bone was contacted. The samples were then subjected to a stress relaxation test using a cylindrical indenter with a diameter of 4 mm where they were compressed to 80% of the original cartilage thickness at a rate of 0.1 mm/s to ensure that the full stress relaxation curve is preserved for accurate characterization. The tissue was then held at a constant strain for 120 seconds. The aggregate modulus (HA) and permeability (κ) of the cartilage tissue was then calculated with units of MPa.

**Histology:**

After biomechanical testing, explants were halved and one half of each sample was used for histological analysis. Briefly, explants were fixed in 10% buffered formalin, decalcified in 10% EDTA, embedded in paraffin, and then sectioned (3 sections of 2-3mm per explant) using a microtome. Tissue sections were stained with hematoxylin and eosin, Safranin O fast green, and Picrosirius

red. Histology was blindly analyzed and scored for cartilage structure, chondrocyte pathology, proteoglycan, collagen integrity, and tidemark based on the OARSI grading system [69]. Total histology score as well as individual category scores were used for correlation analysis.

**Chondrocyte Viability:**

The remaining halves of each explant were assessed for viable chondrocyte density using the fluorescent live cell stain Calcein AM and fluorescent microscopy. Briefly, explants were incubated in 500ul of stain for 25 minutes at 37°C, washed in 500ul of PBS for 5 min at 25°C then mounted in 4% Agarose for imaging. Images were taken at 4X magnification. Green-staining live cells were manually counted through the depth of the tissue, and the area of the tissue was determined. Samples were stored at -20°C until tissue ECM analysis.

**Tissue Extracellular Matrix Analysis:**

Cartilage tissue was resected from the underlying bone, weighed to determine the wet weight, lyophilized, and then reweighed to determine the dry weight of the tissue. Tissues were digested in 1ml of papain (300µg/ml Dithioreitol; and 300µg/ml papain) in papain solution buffer (20mM Sodium phosphate pH 6.8; 1mM EDTA) overnight at 65°C. After papain digestion, tissue proteoglycan (T-GAG) content and collagen content (T-HP) were determined using the dimethylmethylene blue [70] and Hydroxyproline assay [71] respectively, normalized to tissue dry weight, and reported as µg/mg. Briefly, TGAG was measured by diluting tissue digests 1:50 or 1:100 using papain buffer

solution to a total volume of 500µl for each digest. A total volume 300 µl of DMMB reagent (16 µg/mL DMMB; 3mg/ml Glycine; 2.4mg/ml NaCL; 0.1ml/ml of 0.1 N HCL in ddH2O) was added to 5µl of diluted tissue digestion (1:50 or 1:100) and read at 525nm. T-HP was measured by incubating 50 µl of undiluted tissue digestions in 50 µl of 4N sodium hydroxide overnight at 80°C , adding 450 µl of chloramine-T reagent (1.27g (0.056M) chloramine-T dissolved in 20 ml of 50% n-propanol and brought to 100 ml of acetate-citrate buffer) for 25min, followed to addition of 450 µl Erlich's reagent (15g p-dimethylaminobenzaldehyde dissolved in (2:1v/v) n-propanol/perchloric acid and brought to 100ml with ddH2O) for 25min. The absorbance was read at 550nm.

**Media Analysis:**

Media from all patients were assessed for matrix metalloproteinase (MMP)-1, -2, -9, and -13; general MMP activity; prostaglandinE2 (PGE2); nitric oxide (NO); growth-regulated protein (GRO)-α, interleukin (IL)-6, IL-8, monocyte chemotactic protein (MCP)-1; collagen type II cleavage (C2C); type IIA collagen N-propeptide (PIIANP); and GAG content. Media from patients 1 and 2 were further assessed for CASPASE-3, CYTOCHROME C, poly ADP ribose polymerase (PARP); tumor necrosis factor (TNF)-RI, TNF-RII, and death receptor 5 (DR5). Additionally, media from patients 3-6 were assessed for IL-1β, RANTES, TNF-α; and aggrecan chondroitin suflate 846 (CS846).

Luminex multiplex assays were used to measure concentrations of cytokines (Millipore), MMPs (R&D systems), Death Receptors (Invitrogen), and Apoptosis mediators (Invitrogen). EIA was used to measure PGE2 (Cayman Chemical), and

ELISAs were used to measure C2C and CS846 (IBEX Pharmaceuticals Inc). The Griess reagent was used to indirectly measure NO (Promega Corp.). General MMP activity was measured using a fluorescence assay (Anaspec). Finally, M-GAG was measured using DMMB as previously described for T-GAG.

**Statistical Analysis:**

All data were analyzed using SigmaPlot© 13. Correlation of tissue biomechanical properties, histological scoring, biomechanical properties, tissue viability, and media biomarker concentrations for all test groups were determined using Pearson Correlation analyses with significance set at ($p<0.05$) and $r>0.7$ considered strong correlation, $r=0.4$-$0.69$ considered moderate correlation, and $r=0.2$-$0.39$ considered weak correlation.

**Table 3-1:**

| All | Avg. Age | Age Range | Avg. BMI | BMI Range |
|---|---|---|---|---|
| n=6 | 63.58166667 | 56.52-75.61 | 34.33333333 | 28-45 |
| **Male** | | | | |
| n=4 | 66.0975 | 57.96-75.61 | 33.25 | 28-39 |
| **Female** | | | | |
| n=2 | 58.55 | 56.96-60.58 | 36.5 | 28-45 |

## CHAPTER 4: Results

### Histology

Total Histological Scores*:*

Total HISTO score correlations can be found in Table 4-1 and the interrelationships between Total HISTO, $H_A$, κ, TPF, and VCD are represented in Figure 4-1. Total HISTO score had a weak negative correlation with $H_A$ and VCD (r=-0.325 and -0.239 respectively). HISTO was also found to have a moderate positive correlation with HP (r=0.478), weak negative correlations with Media GAG and TNF-RII (r=-0.213 and -0.25 respectively), and a weak positive correlation with CS846 (r=0.303).

Specific Histology Category Scores:

Histology Category correlations can be found in Table 4-2 and the interrelationships between the specific categories are represented in Figure 4-2.Although few correlations were found with total HISTO score, specific histology categories were found to correlate with biomechanical and biochemical properties, and OA biomarkers. Tidemark had weak negative correlations with $H_A$, TPF, and VCD (r=-0.236, -0.222, and -0.371 respectively). Tidemark also had a moderate positive correlation with Tissue GAG (r=0.418), weak positive correlations with IL-6, IL-8, MCP-1, and MMP-2, and weak negative correlations with MMP-1, MMP-9, and MMP-13. Cartilage structure had a weak negative correlation with $H_A$ and VCD (r=-0.396 and -0.297 respectively), as well as with tissue GAG and MMP-1 (r=-0.212 and -0.232 respectively). A moderate positive

correlation was also found between cartilage structure and HP (r=0.513). Chondrocyte score had negative correlations with $H_A$ and VCD (r=-0.257 and -0.202), with tissue GAG and Media GAG (r=-0.234 and r=-0.209 respectively), and a moderate positive correlation with HP (r=0.475). Collagen had weak negative correlations with $H_A$ (r=-0.213), and with tissue GAG and media GAG (r=-0.337, and -0.254 respectively), and a moderate positive correlation with HP (r=0.454). Interestingly proteoglycan score did not correlate with any biomechanical property or VCD. However proteoglycan score had weak negative correlations with tissue GAG and media GAG (r=-0.396 and -0.322 respectively), and a moderate positive correlation with HP (r=0.497).

**Viable Cell Density**

VCD correlations can be found in Table 4-1 and the interrelationships between VCD, $H_A$, κ, TPF, and Total HISTO are represented in Figure 4-1. VCD correlated with many biomechanical and structural properties of the tissue. A moderate positive correlation was found between VCD and $H_A$ (r=0.522), weak positive correlations were found between VCD and κ and TPF (r=0.208 and 0.338 respectively), and a weak negative correlation was found between VCD and total HISTO (r=-0.239). VCD also correlated with a various biomarkers of inflammation, degradation, and apoptosis. Specifically, VCD had a moderate negative correlation with cytokines IL-6, IL-8, and MCP-1 (r= -0.569, -0.574, and -0.567 respectively), and a weak positive correlation to GRO-α (r= -0.302). VCD had a moderate positive correlation with MMP-9 and MMP-13 (r=0.586 and 0.531 respectively), a weak positive correlation with MMP-1 (r=0.397), and a moderate

negative correlation was found between VCD and MMP-2 (r=-0.524). However, no correlation was found between VCD and MMP activity. VCD was also found to have weak negative correlations with CASPASE-3 and $PGE_2$ (r=-0.279 and -0.22 respectively). There were no correlations observed between VCD and death receptors. Interestingly, no correlations were found between VCD and biochemical properties or ECM biomarkers.

**Biomechanical**

Tissue Puncture Force (TPF):

TPF correlations can be found in Table 4-1 and the interrelationships between TPF, $H_A$, κ, Total HISTO, and VCD are represented in Figure 4-1. TPF had a weak negative correlation with κ (r=-0.29), and a weak positive correlation with $H_A$ (r=0.224). Moderate positive correlations were found between TPF and media GAG and CS846 (r=0.549 and 0.49 respectively). Additionally, TPF correlated with a variety of OA biomarkers. Specifically, TPF had moderate negative correlations with IL-6, IL-8, MCP-1, and MMP-2 (r=-0.496, -0.511, -0.459, and -0.425 respectively), and weak negative correlations with GRO-α, RANTES, and NO (r=-0.289, -0.332, and -0.32 respectively). A strong positive correlation was found between TPF and MMP-13 (r=0.716). Finally, TPF had a moderate positive correlation with MMP-9 (r=0.424) and a weak positive correlation with MMP activity (r=0.215).

Aggregate Modulus ($H_A$):

$H_A$ correlations can be found in Table 4-1 and the interrelationships between $H_A$, κ, TPF, Total HISTO, and VCD are represented in Figure 4-1. In

addition to correlating with HISTO, VCD, and TPF as stated previously, $H_A$ correlated with ECM, inflammatory, and degradative biomarkers. $H_A$ had a moderate negative correlation with CS846 (r=-0.402), and weak negative correlations with IL-6, IL-8, MCP-1, MMP-2, and $PGE_2$ (r=-0.36, -0.356, -0.356, -0.337, and -0.201 respectively). $H_A$ also had weak positive correlations with MMP-1, MMP-9, and MMP-13 (r=0.338, 0.33, and 0.352 respectively). Interestingly, $H_A$ did not correlate with any biochemical properties of the tissue.

Permeability (κ):

K score correlations can be found in Table 4-1 and the interrelationships between κ, $H_A$, Total HISTO, TPF, and VCD are represented in Figure 4-1. There were very few weak correlations observed between κ and biomechanics, biochemistry, and OA biomarkers. In addition to correlating with TPF and VCD as stated previously, κ had weak positive correlations with RANTES and TNF-RI (r=0.251 and 0.269 respectively). No other correlations were found.

**ECM Content**

The correlation values for TGAG, MGAG, and HP can be found in Table 4-3. Interrelationships between ECM biomarkers can be found in Figure 4-3.

Tissue GAG:

Tissue GAG had a moderate negative correlation with HP (r=-0.488) and a moderate positive correlation with media GAG (r=0.46). Correlations were also found between tissue GAG and a variety of biomarkers. Tissue GAG had moderate positive correlations with CS846 and Cytochrome C (r=0.436 and 0.529 respectively), and a weak positive correlation with $PGE_2$ (r=0.299). A

moderate negative correlation was found between tissue GAG and DR5 (r=-0.4), and weak negative correlations were found with MMP-9, MMP activity, RANTES, and CASPASE-1 (r=-0.32, -0.239, -0.36, and -0.329 respectively).

Media GAG*:*

Media GAG also had a moderate negative correlation with HP (r=-0.406). Additionally, media GAG had weak positive correlations with $PGE_2$, MMP-13, and CS846 (r=0.237, 0.241, and 0.357 respectively), and weak negative correlations with RANTES, Cytochrome C, and DR5 (r=-0.353, -0.261, and -0.261 respectively).

Tissue HP*:*

HP had a weak positive correlation with CS846 and Cytochrome C (r=0.337 and 0.308 respectively). No other correlations were found.

**Biomarkers**

Inflammatory indicators:

NO and $PGE_2$ correlations can be found in Table 4-4 and the interrelationships between NO and $PGE_2$ are represented in Figure 4-4. NO had a weak positive correlation to TNF-RII (r= 0.333), a moderate positive correlation to MMP-2, IL-6, IL-8, GRO-α, MCP-1 (r= 0.421, 0.55, 0.549, 0.438, and 0.47 respectively), and a weak to moderate negative correlation to MMP-9, MMP-13, and CS846 (r= -0.396, -0.465, and -0.393 respectively). $PGE_2$ had a weak positive correlation to Media GAG, MMP activity, GRO-α, IL-6, IL-8, MCP-1, and TNF-RII (r= 0.237, 0.248, 0.365, 0.32, 0.329, 0.302, and 0.337 respectively).

MMPs:

MMP correlations can be found in Table 4-5 and the interrelationships between MMPs are represented in Figure 4-5. MMP-1, MMP-9, and MMP-13 all had a weak to moderate positive correlation to general MMP activity (r= 0.363, 0.541, and 0.247 respectively). General MMP activity had a weak to moderate positive correlation with, RANTES, CASPASE-3, TNF-RI, TNF-RII, and DR5 (r= 0.275, 0.323, 0.559, 0.452, and 0.552 respectively). MMP-1 had a weak positive correlation with MMP-9 and RANTES (r= 0.251 and 0.393 respectively), and a weak negative correlation to MCP-1 and CS846 (r= -0.202 and -0.355 respectively). MMP-2 had a weak to moderate positive correlation to DR5, GRO-α, IL-6, IL-8, and MCP-1 (r= 0.255, 0.307, 0.51, 0.513, and 0.49 respectively), and a moderate negative correlation to MMP-9 and MMP-13 (r= -0.435 and -0.424 respectively). MMP-9 and MMP-13 had a weak to moderate positive correlation to each other (r= 0.61), TNF-RI (r= 0.544 for MMP-9 and 0.3 for MMP-13), and TNF-RII (r= 0.445 for MMP-9 and 0.351for MMP-13), and a moderate negative correlation to IL-6 (r= -0.495 for MMP-9 and -0.644 for MMP-13), IL-8 (r=-0.507 for MMP-9 and -0.665 for MMP-13), and MCP-1 (r= -0.497 for MMP-9 and -0.61 for MMP-13). MMP-9 had a moderate positive correlation with CASPASE-3, Cytochrome C, and RANTES (r= 0.534, 0.469, and 0.517 respectively), and a strong positive correlation to DR5 (r= 0.738). MMP-13 had a weak to moderate positive correlation to Media GAG, CS846, and DR5 (r= 0.241, 0.559, and 0.352 respectively), and a moderate negative correlation to GRO-α and RANTES (r= -0.411 and -0.379 respectively).

Cytokines:

Cytokine correlations can be found in Table 4-6 and the interrelationships between Cytokines are represented in Figure 4-6. IL-1β and TNF-α were below the detection limit of their respective assay and therefore were not included in these results. GRO-α, IL-6, IL-8, and MCP-1 had a strong positive correlation to each other (r≥ 0.731). RANTES did not correlate with GRO-α, IL-6, IL-8, or MCP-1. GRO-α, IL-6, and MCP-1 had a weak to moderate positive correlation to TNF-RII (r= 0.433, 0.271, and 0.261 respectively). GRO-α had a weak to moderate positive correlation to PIIANP (r= 0.282), CASPASE-3 (r=0.254), TNF-RI (r= 0.433), and DR5 (r= 0.43), and a weak negative correlation to CS846 (r= -0.27). RANTES had a weak negative correlation with Tissue GAG (r= -0.36) and Media GAG (r= -0.353).

Apoptosis Markers and Death Receptors:

Apoptosis Marker correlations can be found in Table 4-7 and Death Receptor correlations can be found in Table 4-8. TNF-RI had a moderate to strong positive correlation to TNF-RII and DR5 (r= 0.678 and 0.732 respectively), and TNF-RII had a strong positive correlation to DR5 (r= 0.719). CASPASE-3 had a weak to moderate positive correlation to TNF-RI, TNF-RII, and DR5 (r= 0.289, 0.419, and 0.635 respectively), and a strong positive correlation to Cytochrome C (r= 0.769). Cytochrome C had a moderate positive correlation with DR5 (r= 0.529). Cytochrome C and DR5 had a weak negative correlation with

Media GAG (r=-0.261 for both). TNF-RI had a weak negative correlation with C2C (r= 0.3).

**Table 4-1:**

| Biomarker Prodcution vs. Tissue Mechanical and Structural Properties | | | | | |
|---|---|---|---|---|---|
| | $H_A$ | κ | TPF | HISTO | VCD |
| **Biomechanics** | R | R | R | R | R |
| $H_A$ | | | 0.224 | -0.325 | 0.522 |
| κ | | | -0.29 | | 0.208 |
| TPF | | | | | 0.338 |
| **Total Histo** | | | | | -0.239 |
| **Cytokine** | | | | | |
| GRO-α | | | -0.289 | | -0.302 |
| IL-6 | -0.36 | | -0.496 | | -0.569 |
| IL-8 | -0.356 | | -0.511 | | -0.574 |
| MCP-1 | -0.356 | | -0.459 | | -0.567 |
| RANTES | | 0.251 | -0.332 | | |
| **MMPS** | | | | | |
| MMP-1 | 0.338 | | | | 0.397 |
| MMP-2 | -0.337 | | -0.425 | | -0.524 |
| MMP-9 | 0.33 | | 0.424 | | 0.586 |
| MMP-13 | 0.352 | | 0.716 | | 0.531 |
| ACTIVITY | | | 0.215 | | |
| **Death Receptors** | | | | | |
| TNF-RI | | 0.269 | | | |
| TNF-RII | | | | -0.25 | |
| DR5 | | | | | |
| **Apoptosis** | | | | | |
| CASPASE-3 | | | | | -0.279 |
| CYTOCHROME C | | | | | |
| **Inflammation** | | | | | |
| NO | | | -0.32 | | |
| PGE2 | -0.201 | | | | -0.22 |
| **Extra Cellular Matrix** | | | | | |
| HP | | | | 0.478 | |
| TISSUE GAG | | | | | |
| MEDIA GAG | | | 0.549 | -0.213 | |
| CS846 | -0.402 | | 0.49 | 0.303 | |

**Figure 4-1:**

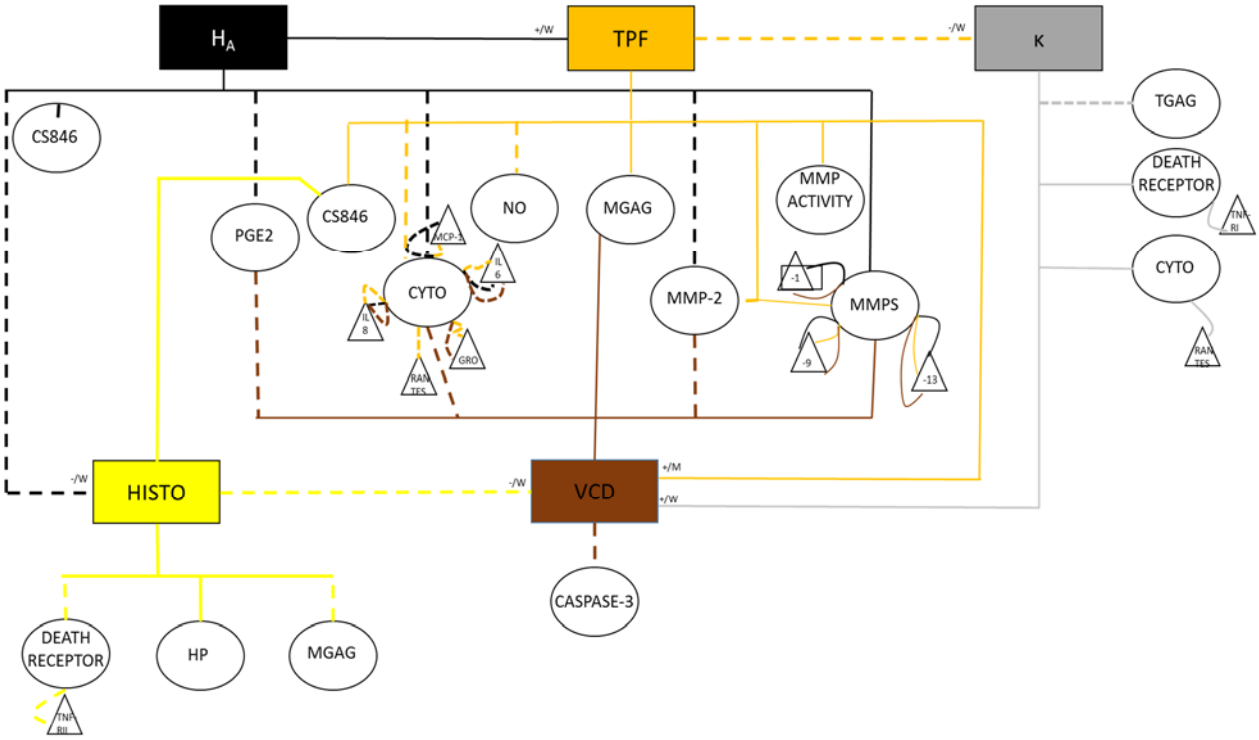
H_A
+/W
TPF
-/W
K
CS846
PGE2
CS846
NO
MGAG
MMP ACTIVITY
MCP-1
IL 6
CYTO
IL 8
GRO
RAN TES
MMP-2
-1
MMPS
-9
-13
TGAG
DEATH RECEPTOR
TNF RI
CYTO
RAN TES
-/W
HISTO
-/W
VCD
+/M
+/W
CASPASE-3
DEATH RECEPTOR
TNF RII
HP
MGAG

**Table 4-2:**

| Biomarker Prodcution vs. Tissue Mechanical and Structural Properties | | | | | | |
|---|---|---|---|---|---|---|
| | CART STRUCTURE | CHONDROCYTES | PROTEOGLYCAN | COLLAGEN | TIDEMARK | TOTAL |
| **Biomarkers** | **R** | **R** | **R** | **R** | **R** | **R** |
| **Cytokine** | | | | | | |
| IL-6 | | | | | 0.351 | |
| IL-8 | | | | | 0.34 | |
| MCP-1 | | | | | 0.361 | |
| **MMPS** | | | | | | |
| MMP-1 | -0.232 | | | | -0.266 | |
| MMP-2 | | | | | 0.324 | |
| MMP-9 | | | | | -0.256 | |
| MMP-13 | | | | | -0.236 | |
| **Extra Cellular Matrix** | | | | | | |
| HP | 0.513 | 0.457 | 0.497 | 0.454 | | 0.478 |
| TISSUE GAG | -0.212 | -0.234 | -0.396 | -0.337 | 0.418 | -0.199 |
| MEDIA GAG | | -0.209 | -0.322 | -0.254 | | -0.213 |
| **Biomechanics** | | | | | | |
| AG | -0.396 | -0.257 | | -0.213 | -0.236 | -0.325 |
| TPF | | | | | -0.222 | |
| **VCD** | -0.297 | -0.202 | | | -0.371 | -0.239 |

**Figure 4-2:**

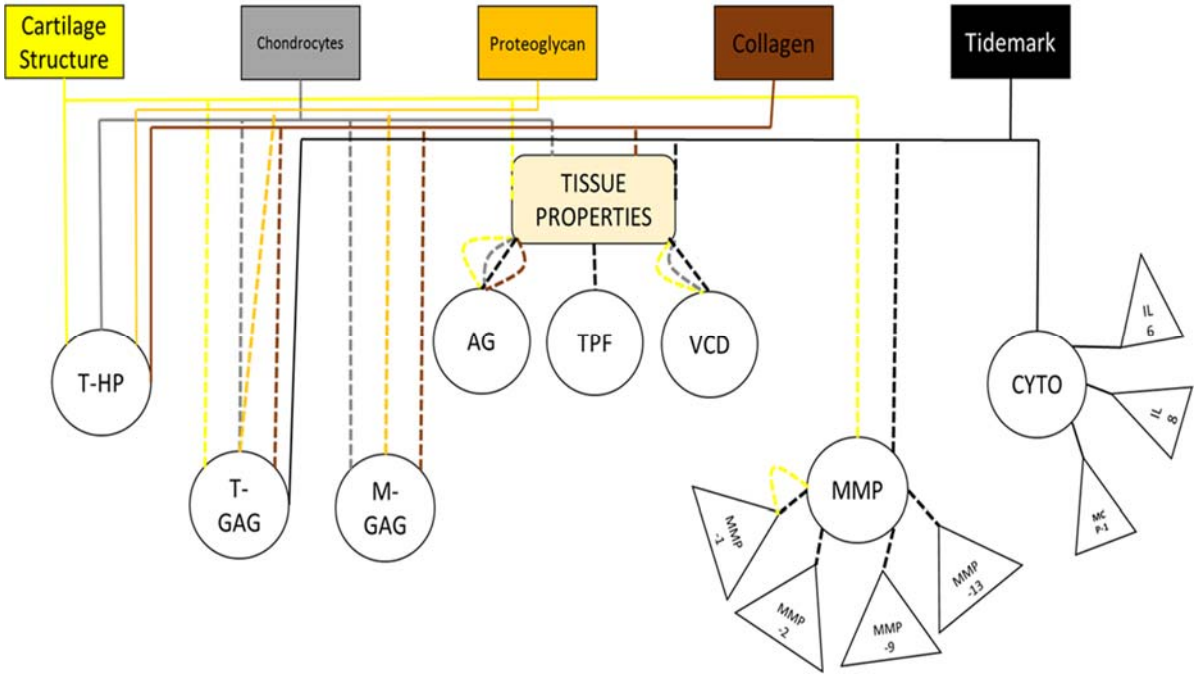
Cartilage Structure
Chondrocytes
Proteoglycan
Collagen
Tidemark
TISSUE PROPERTIES
T-HP
T-GAG
M-GAG
AG
TPF
VCD
CYTO
IL 6
IL 8
MCP-1
MMP
MMP -1
MMP -2
MMP -9
MMP -13

**Table 4-3:**

| | TGAG | MGAG | CS846 | T-HP |
|---|---|---|---|---|
| **ECM** | **R** | **R** | **R** | **R** |
| TISSUE GAG | | 0.46 | 0.436 | -0.488 |
| MEDIA GAG | | | 0.357 | -0.406 |
| CS846 | | | | 0.337 |
| **Cytokine** | | | | |
| GRO-α | | | -0.27 | |
| RANTES | -0.36 | -0.353 | | |
| **MMPS** | | | | |
| MMP-1 | | | -0.355 | |
| MMP-9 | -0.32 | | | |
| MMP-13 | | 0.241 | 0.559 | |
| ACTIVITY | -0.239 | | | |
| **Death Receptors** | | | | |
| DR5 | -0.4 | -0.261 | | |
| **Apoptosis** | | | | |
| CASPASE-3 | -0.329 | | | |
| CYTOCHROME C | 0.529 | -0.261 | | 0.308 |
| **Inflammation** | | | | |
| NO | | | -0.393 | |
| PGE2 | 0.299 | 0.237 | | |

**Figure 4-3:**

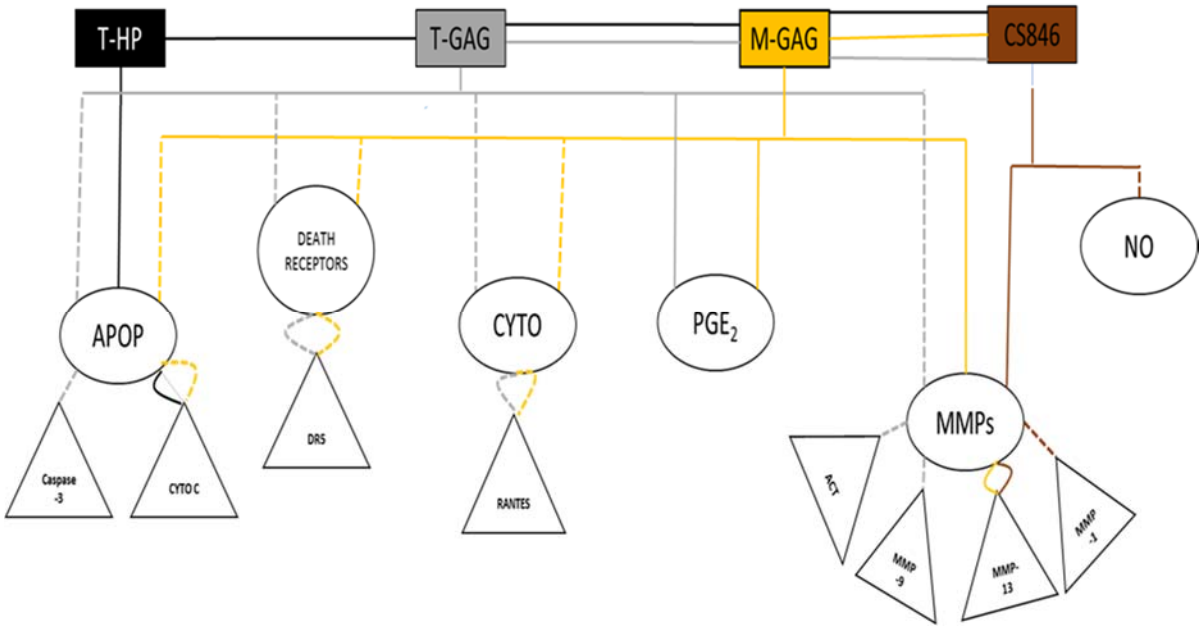

**Table 4-4:**

| | NO | PGE2 |
|---|---|---|
| **Cytokine** | **R** | **R** |
| GRO-α | 0.438 | 0.365 |
| IL-6 | 0.55 | 0.32 |
| IL-8 | 0.549 | 0.329 |
| MCP-1 | 0.47 | 0.302 |
| **MMPS** | | |
| MMP-2 | 0.421 | |
| MMP-9 | -0.396 | |
| MMP-13 | -0.465 | |
| ACTIVITY | | 0.248 |
| **Death Receptors** | | |
| TNF-RII | 0.333 | 0.337 |
| **Extra Cellular Matrix** | | |
| TISSUE GAG | | 0.299 |
| MEDIA GAG | | 0.237 |
| CS846 | -0.393 | |

**Figure 4-4:**

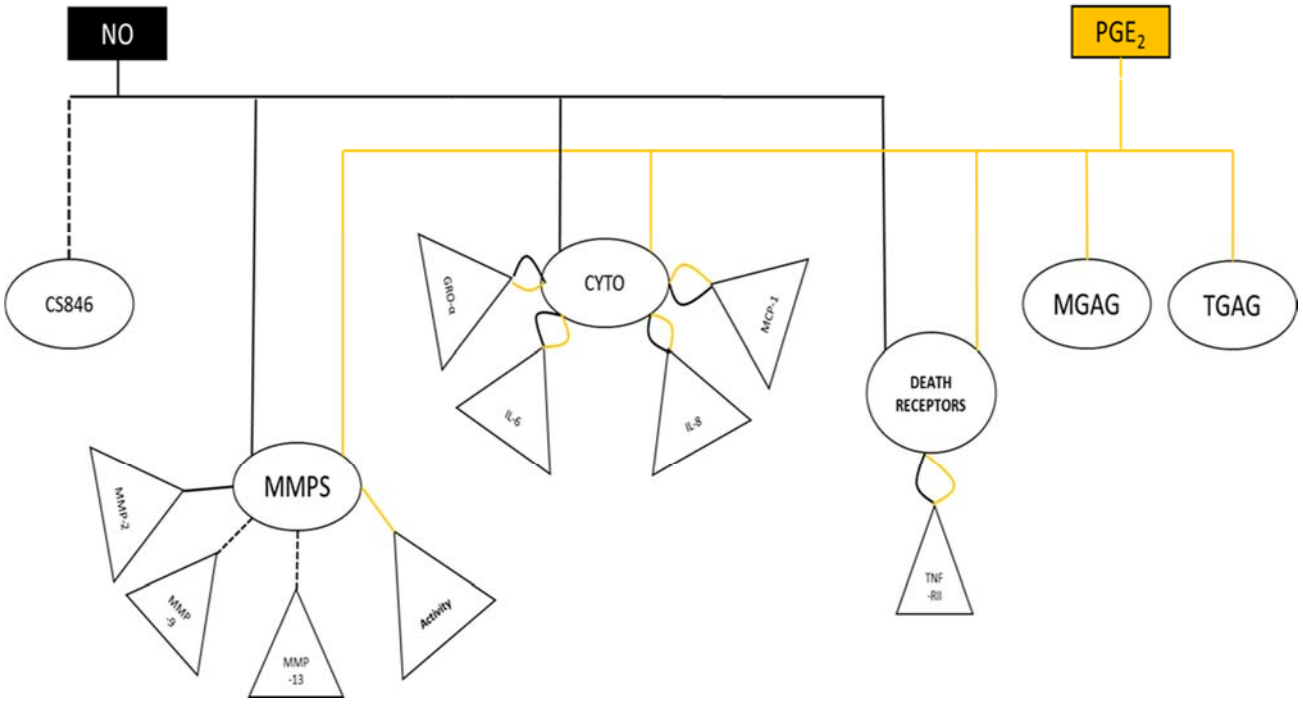

**Table 4-5:**

| | MMP-1 | MMP-2 | MMP-9 | MMP-13 | MMP ACT |
|---|---|---|---|---|---|
| **MMPS** | **R** | **R** | **R** | **R** | **R** |
| MMP-1 | | | 0.251 | | 0.363 |
| MMP-2 | | | -0.436 | -0.424 | |
| MMP-9 | | | | 0.61 | 0.541 |
| MMP-13 | | | | | 0.247 |
| **Cytokines** | | | | | |
| GRO-α | | 0.307 | | -0.411 | |
| IL-6 | | 0.51 | -0.495 | -0.644 | |
| IL-8 | | 0.513 | -0.507 | -0.665 | |
| MCP-1 | -0.202 | 0.49 | -0.497 | -0.61 | |
| RANTES | 0.393 | | 0.517 | -0.379 | 0.466 |
| **Death Receptors** | | | | | |
| TNF-RI | | | 0.544 | 0.3 | 0.559 |
| TNF-RII | | | 0.445 | 0.351 | 0.452 |
| DR5 | | 0.255 | 0.738 | 0.352 | 0.552 |
| **Apoptosis** | | | | | |
| CASPASE-3 | | | 0.534 | | 0.323 |
| CYTOCHROME C | | | 0.469 | | |
| **Inflammation** | | | | | |
| NO | | 0.421 | -0.396 | -0.465 | |
| PGE2 | | | | | 0.248 |
| **Extra Cellular Matrix** | | | | | |
| TISSUE GAG | | | -0.32 | | |
| MEDIA GAG | | | | 0.241 | -0.239 |
| CS846 | -0.355 | | | 0.559 | |

**Figure 4-5:**

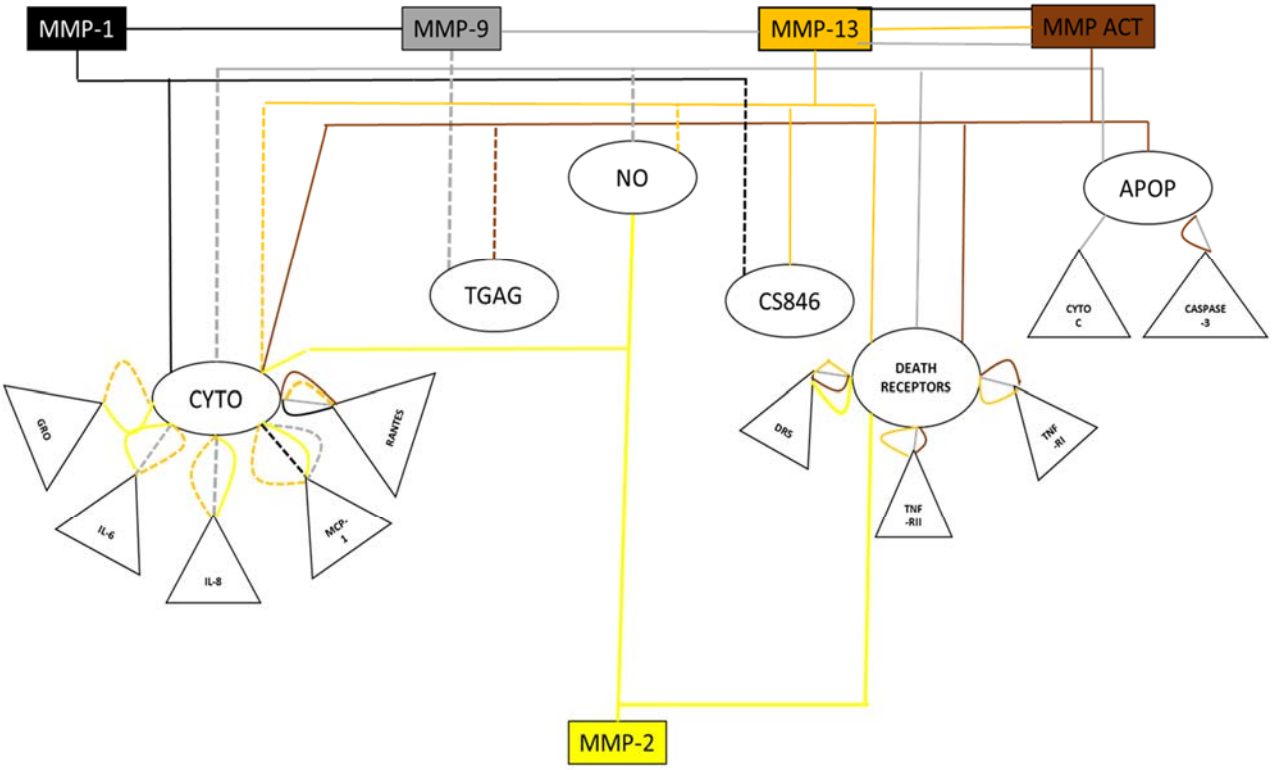
MMP-1
MMP-9
MMP-13
MMP ACT
NO
APOP
TGAG
CS846
CYTO C
CASPASE -3
CYTO
DEATH RECEPTORS
GRO
RANTES
IL-6
MCP-1
IL-8
DRS
TNF -RI
TNF -RII
MMP-2

**Table 4-6:**

| | GRO-α | IL-6 | IL-8 | MCP-1 | RANTES |
|---|---|---|---|---|---|
| **CYTOKINE** | **R** | **R** | **R** | **R** | **R** |
| GRO-α | | 0.765 | 0.763 | 0.731 | |
| IL-6 | | | 0.987 | 0.959 | |
| IL-8 | | | | 0.97 | |
| MCP-1 | | | | | |
| RANTES | | | | | |
| **MMPS** | | | | | |
| MMP-1 | | | | -0.202 | 0.393 |
| MMP-2 | 0.307 | 0.51 | 0.513 | 0.49 | |
| MMP-9 | | -0.495 | -0.507 | -0.497 | 0.517 |
| MMP-13 | -0.411 | -0.644 | -0.665 | -0.61 | -0.379 |
| ACTIVITY | | | | | 0.466 |
| **Death Receptors** | | | | | |
| TNF-RI | 0.433 | | | | |
| TNF-RII | 0.552 | 0.271 | | 0.261 | |
| DR5 | 0.43 | | | | |
| **Inflammation** | | | | | |
| NO | 0.438 | 0.55 | 0.549 | 0.47 | |
| PGE2 | 0.365 | 0.32 | 0.329 | 0.302 | |
| **Apoptosis** | | | | | |
| CASPASE-3 | 0.254 | | | | -0.36 |
| **Extra Cellular Matrix** | | | | | |
| TISSUE GAG | | | | | -0.36 |
| MEDIA GAG | | | | | -0.353 |
| PIIANP | 0.282 | | | | |
| CS846 | -0.27 | | | | |

**Figure 4-6:**

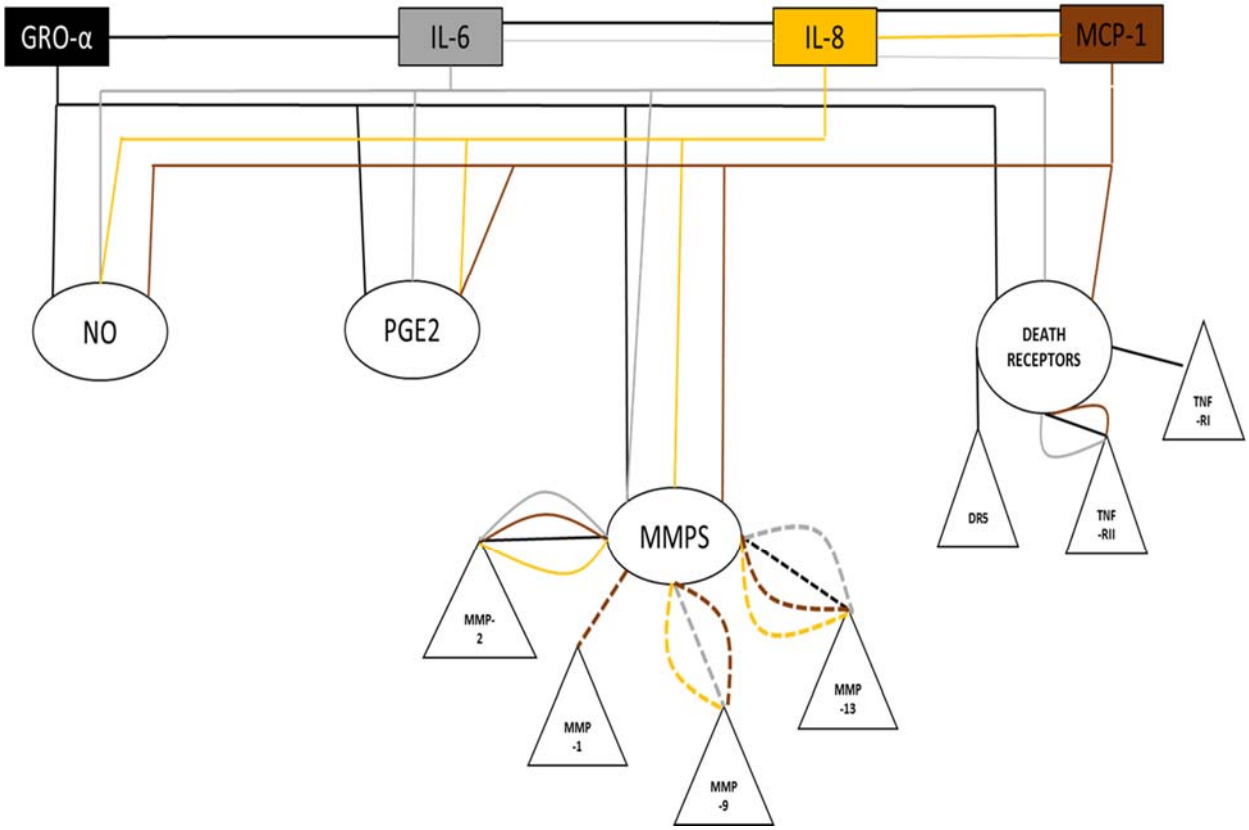


**Table 4-7:**

| | CASPASE-3 | CYTOCHROME C |
|---|---|---|
| **Apoptosis** | **R** | **R** |
| CASPASE-3 | | 0.769 |
| **Cytokine** | | |
| GRO-α | 0.254 | |
| **MMPS** | | |
| MMP-9 | 0.534 | 0.469 |
| ACTIVITY | 0.323 | |
| **Death Receptors** | | |
| TNF-RI | 0.289 | |
| TNF-RII | 0.419 | |
| DR5 | 0.635 | 0.529 |
| **Extra Cellular Matrix** | | |
| TISSUE GAG | -0.329 | 0.529 |
| MEDIA GAG | | -0.261 |
| HP | | 0.308 |

**Table 4-8:**

| Table 9 | TNF-RI | TNF-RII | DR5 |
|---|---|---|---|
| **Death Receptors** | **R** | **R** | **R** |
| TNF-RI | | 0.678 | 0.732 |
| TNF-RII | | | 0.719 |
| **Cytokine** | | | |
| GRO-α | 0.433 | 0.552 | 0.43 |
| IL-6 | | 0.271 | |
| MCP-1 | | 0.261 | |
| **MMPS** | | | |
| MMP-2 | | | 0.255 |
| MMP-9 | 0.445 | 0.544 | 0.738 |
| MMP-13 | 0.351 | 0.3 | 0.352 |
| ACTIVITY | 0.452 | 0.559 | 0.552 |
| **Apoptosis** | | | |
| CASPASE-3 | 0.289 | 0.519 | 0.635 |
| CYTOCHROME C | | | 0.529 |
| **Inflammation** | | | |
| NO | | 0.333 | |
| PGE2 | | 0.337 | |
| **ECM** | | | |
| TGAG | | | -0.4 |
| MGAG | | | -0.261 |
| C2C | -0.31 | | |

## CHAPTER 5: Discussion

In this study, we found an extensive network of interrelationships between the changes in biomechanical properties, biochemical composition, histological grade, and chondrocyte viability in osteoarthritic knee cartilage. Previous studies have reported decreases in cell viability and increases in apoptosis, and important correlations between chondrocyte viability, surface receptor binding of native collagen type II, and histopathology scores in OA cartilage [37, 72-75]. In the present study, chondrocyte viability negatively correlated with total histology score, specifically negative correlations were noted between VCD and cartilage structure score, chondrocyte pathology score, and tidemark score. Additionally, we show chondrocyte viability had a moderate positive correlation with $H_A$, and a weaker positive correlation with permeability. Together, these data support previous reports indicating chondrocyte death as a contributing factor in the disruptive processes of OA. However, no correlations were found between VCD and ECM content, suggesting that chondrocyte death may be a consequence of these events rather than a primary driver of them.

Decreases in tensile, compressive, and shear properties, and collagen and proteoglycan content and correlations between mechanical, biochemical, and histological changes in OA cartilage have also been previously reported [5, 33, 34, 76]. In agreement with these findings, we showed $H_A$ to negatively correlate with CS846 and total histology score. Specifically, $H_A$ negatively

correlated with cartilage structure, chondrocyte pathology, collagen, and tidemark score. Although a negative correlation was found between $H_A$ and CS846 in the media, no correlation was found between $H_A$ and Tissue GAG, Media GAG, or proteoglycan histology score. These results are inconsistent with previous literature in which increasing evidence indicate compressive properties to be related to proteoglycan content [5, 10]. Furthermore, total histology score was positively correlated with tissue HP content and negatively correlated to Media GAG. The positive correlation with tissue HP, negative correlations with Media GAG, and negative correlations with Tissue GAG were replicated across cartilage structure, chondrocyte pathology, proteoglycan, and collagen scores. Tissue GAG had a moderately positive correlation with tidemark score possibly contributing to the negligible correlation found with the total histology score.

Using a needle probe test, TPF, or the force required to break through the superficial most layer of the cartilage, or lamina splendens was measured. Disruption of the densely packed collagen meshwork in the lamina splendens and superficial zone appears early in OA and is shown to reduce the ability of cartilage to resist mechanical forces [1, 2, 5, 8, 11, 17, 30]. Thus, TPF is an indirect indictor of the integrity of the surface structure. Interestingly, we showed that TPF positively correlated with Media GAG, CS846, and to a lesser extent VCD and $H_A$, and negatively correlated to permeability. No correlation was found between TPF and total histology score, and further analysis showed only a weak negative correlation with tidemark score. Using a needle probe limits the measurement of TPF to a very small area, and since we only obtained one

measurement in the center of the tissue, our data do not necessarily represent the tissue as a whole, and therefore may explain the discrepancies in our results. However, surface integrity was not scored separately histologically and therefore further investigation is needed to determine whether TPF correlates with surface integrity of the tissue.

While these data clearly indicate interrelationships between biomechanics, ECM biochemical content, histology, and VCD, direct measurement of these properties *in vivo* are highly expensive, invasive, or nonexistent. Changes in mechanical and structural properties and biochemical composition of the ECM is shown to affect the cellular function of OA chondrocytes [4]. However, studies have focused on the correlation of only a limited number of biomarkers to these properties individually. Abnormal static or dynamic loading of chondrocytes and cartilage tissue induces cytokine, MMP, NO, and $PGE_2$ which in turn have been shown to induce changes in compressive, tensile, and shear properties [5, 10, 27]. Therefore, we investigated relationships of these and other known OA biomarkers with the physical properties of OA cartilage, as well as their relationships to each other.

The data indicates a correlation between $H_A$, TPF, and VCD and a variety of biomarkers. Specifically, $H_A$, TPF, and VCD all showed negative correlations with cytokine production, most notably GRO-α, IL-6, IL-8, and MCP-1. These findings are consistent with previous studies in which increased cytokine production was associated with increased severity of OA [45, 46, 48, 49, 77]. Furthermore, suprarphysiological mechanical stress has been shown to stimulate

production of cytokines [26, 50]. $H_A$, TPF, and VCD also showed a positive correlation with MMP-1, MMP-9, and MMP-13, but a negative correlation with MMP-2. Permeability and total histology score did not seem to correlate to biomarkers, however the individual tidemark histology score did correlate positively with cytokines and MMP-2, and negatively with MMP-1, MMP-9, and MMP-13. In contrast, studies report increased expression of MMPs in OA cartilage with increasing histological grades [58, 60, 63, 78], and increased production of MMPs following mechanical stress [27, 50]. Furthermore, MMP-13 production is reported to be associated with increased calcification and tidemark advancement [79], and ablation of MMP-13 stabilizes the ECM and decreases chondrocyte death [54]. Although a variation of models were used in these study such as, monolayer culture of isolated human OA chondrocytes [58], or osteochondral explants from normal or OA patients [60, 78]. These differences in models may contribute to the differences in MMP production.

The data also indicates a correlation between tissue properties and biomarkers of inflammation, apoptosis, and death receptors. Specifically, a positive correlation was found between permeability and TNF-RI, and a negative correlation between total histology score and TNF-RII. Consistent with previous studies showing direct relationships between CASPASE-3 [80]and chondrocyte density, we showed that VCD negatively correlated with CASPASE-3. However, VCD did not correlate with Cytochrome C, another apoptotic marker, or NO which has previously been proposed to be associated with apoptosis and chondrocyte death [8, 37, 56]. Finally, VCD and $H_A$ negatively correlated to

$PGE_2$. It has been reported that NO and $PGE_2$ production increases in response to mechanical stimulation of cartilage [27, 51, 81], therefore these findings suggest that inflammation may be a direct response of increased mechanical stress on the cartilage tissue as a result of poor tissue mechanical properties.

In an attempt to further characterize biomarker production, we analyzed correlations between the biomarkers. We found that cytokines and MMPs were negatively correlated to each other and in most cases, opposite correlations were found with other biomarkers. GRO-α, IL-6, IL-8, and MCP-1 were shown to negatively correlate with MMP-9 and MMP-13, and positively correlate with MMP-2. These results are consistent with previous studies in our lab in which stimulation of normal canine cartilage, synovium, or fatpad with IL-6, IL-8, or MCP-1 or OA osteochondral tissue with IL-1β or TNF-α did not increase production of MMPs or MMP activity (data not published). In a recent study comparing disease modifying OA drugs (DMOADs), lower levels of baseline MMP-1 and higher levels of baseline IL-6 were associated with cartilage volume loss in both licofelone and naproxen groups. Furthermore, increases in MMP-1 and MMP-13 and decreases in IL-6 were associated with cartilage volume loss in both treatment groups [82]. However in contrast to this finding, it has been well reported that cytokines such as the ones in this study upregulate MMP expression in normal and OA chondrocytes [4, 50, 53]. MCP-1, GRO-α, and RANTES increased production of MMP-3, which has been shown to signal production of other MMPs, and IL-8 was shown to induce MMP-13 in normal and OA chondrocytes [61, 62]. MMP-2, MMP-3, MMP-9, and MMP-13 expression and

activity was induced by IL-1β and IL-6 in mice calvariae [83]. MMP-1, and MMP-13 was increased in normal and OA chondrocytes stimulated with TNF-α and OA chondrocytes stimulated by IL-6 [59]. Although in an earlier study, TNF-α induced MMP-1 and MMP-13 production was significantly higher in OA chondrocytes from unaffected regions compared to those near lesions [58]. One hypothesis for these findings could be the dual pro-inflammatory and repair roles of these cytokines [4, 19, 55], in which they could be promoting repair mechanisms in earlier stages and contributing to degradative processes in the advanced stages of OA.

It is also important to note that GRO-α, IL-6, IL-8, and MCP-1, but not RANTES, seemed to correlate as a group. IL-1β and TNF-α stimulates the production of subsequent proinflammatory cytokines in cartilage and synovial tissue [7, 19]. Furthermore, in combination with each other, proinflammatory cytokines, such as the ones studies here, are shown to have synergistic effects on the production of matrix degrading enzymes and inflammatory molecules [7, 84]. Here we showed that GRO-α, IL-6, IL-8, and MCP-1 positively correlated with TNF-RI, NO, and $PGE_2$. Similar relationships between cytokines and inflammatory markers are reported throughout the literature [56]. IL-1β induced expression of NO synthase in chondrocytes [26], and $PGE_2$ production was increased by TNF-α stimulation of synovial fibroblast, addition of IL-8 enhanced this increase [84]. Furthermore, incubation of chondrocytes with $PGE_2$ induced expression of IL-6 [85]. Although production of IL-1β and TNF-α were below

detection levels, these cytokines may be produced primarily by synovial tissue which then signals the production of these cytokines by the cartilage.

Similarly, MMP-1, and to a greater extent MMP-9 and MMP-13 seemed to correlate as a collective group. Interestingly, MMP-2 concentration negatively correlated to the other MMPs. Previously, coexpression correlations were observed between MMP-2, MMP-9, and MMP-13, and MMP-1 and MMP-3 in human normal and OA chondrocytes with a negative relationship observed between the two groups [86]. However, other studies have shown different patterns of coproduction or coexpression of MMPs in which increases of MMP-1 and MMP-13 [58, 82], MMP-2, MMP-9, and MMP-9 [27], or MMP-2, MMP-3, and MMP-13 [83] were associated with OA progression or following cytokine or compression stimulation. There are also conflicting reports of whether MMP expression is localized to the superficial zone [63] or the deep zone [60, 78]. Together, the data from this study and previous studies suggest varying patterns of coregulation in MMP production which could possibly be associated with the different stages of OA or the intermittent pattern of active and quiescent phases of disease progression as suggested by Tetlow et el [63].

Additionally, MMP-9 and MMP-13 were shown to correlate positively with death receptors and negatively with NO, and MMP-2 was shown to positively correlate to NO. In contrast, production of MMPs have been reported to parallel production of NO as well as $PGE_2$ in response to mechanical stimulus, as well as in OA cartilage [4, 43, 55].Mechanical stimulation induced production of NO and MMP-2, MMP-9, and MMP-13 in both costal and articular cartilage explants

[27].Along with MMP concentrations, we have shown that MMP activity was also correlated positively with death receptors, as wells as CASPASE-3 and $PGE_2$.

Finally, we show that biomarkers of inflammation correlate with other biomarkers as well, but to a lesser extent. Individually, NO correlated negatively with MMPs and CS846, and $PGE_2$ positively correlated to Tissue and Media GAG. However, NO and $PGE_2$ were not correlated with one another and only shared similar correlations with cytokines and TNF-RII. These results are also seen in correlations with tissue properties as well, $H_A$ and VCD correlated to $PGE_2$ but not NO, and TPF correlated to NO but not $PGE_2$. It has been previously shown that $PGE_2$ production significantly increases with inhibition of NO and significantly decreases with NO stimulation of OA cartilage [87]. These findings suggest different metabolic roles of NO and $PGE_2$ in OA and that production of NO may play a role in inhibiting the production of $PGE_2$. Therefore, profiles of varying levels of these inflammatory molecules may have the potential to characterize different stages of OA or different OA subgroups (ex. PTOA, age-associated, or obesity-associated).

These results are limited to the production of one area of cartilage tissue and therefore do not represent the production of the cartilage tissue as a whole, nor the entire joint. Furthermore, the level of biomarkers are limited to the amount of protein released into the media during culture. With the exception of tissue glycosaminoglycan content and hydroxyproline content, the expression of these biomarkers within the tissue were not studied. Finally, osteochondral samples were obtained from a small patient group with end-stage joints. Therefore,

cartilage exhibiting less degradation do not truly reflect earlier stages of disease as they have been exposed to end-stage environment within the joint.

In conclusion, we have demonstrated that changes in biomechanical, biochemical, and histological properties of OA cartilage are interrelated, and that these tissue properties are correlated to numerous biomarkers of inflammation, degradation, and cell viability. Most notably, we show that cytokines and MMPs correlated to a wide range of tissue properties and other biomarkers. Therefore, panels including both cytokines and MMPs have a potential to characterize cartilage structural and mechanical integrity, and knee joint health in the clinical setting. Furthermore, differing patterns of production of these biomarkers may begin to distinguish between subgroups of OA or even different stages during disease progression. Further *in vitro* and *in vivo* validation of the relationships between these biomarkers and tissue properties are needed in order to identify more distinguished patterns of production during OA. Understanding the correlation between tissue properties and the production of biomarkers by the tissue has to potential to significantly increase our understanding of how OA develops and progresses clinically. As our understanding of the factors that contribute to the development and progression of the disease increases, our ability to detect OA development earlier and design new disease modifying drugs increases as well.

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