PATHOBIOLOGICAL ANALYSIS OF DEVELOPMENTAL DYSPLASIA OF THE HIP AND ITS PROGRESSION TO OSTEOARTHRITIS

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by

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

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a candidate for the degree of Doctor of Philosophy,

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

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DEDICATION

I am dedicating this work to my friends and family who have constantly supported me through this journey. First, my close friends, Dakota Smith, Parks Boeschen, Price Meentemeyer, Clayton Birkes, and many more, who have continually pushed me to be the best I can be and provided the much-needed support throughout these past 4+ years. Next, my countless family members who always supported me and allowed me to explore my own interests without feeling pressured to take a more “typical” route. Third, my teachers and professors in several different disciplines which showed me how learning and expanding my knowledge was exciting and something I should strive for each day. Finally, William Woods University and the athletics program for showing me how to be a leader and providing a wonderful environment to learn inside and outside the classroom.

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PATHOBIOLOGICAL ANALYSIS OF DEVELOPMENTAL DYSPLASIA OF THE HIP AND ITS PROGRESSION TO OSTEOARTHRITIS

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ABSTRACT

Developmental dysplasia of the hip (DDH) is recognized as one of the leading causes of early-onset hip osteoarthritis and a primary reason for undergoing total hip arthroplasty prior to 50 years of age. Even with an increase in awareness and focus on infant screening and early diagnosis, approximately 15-25% of DDH cases remain undiagnosed into early adulthood when prevention is no longer possible, interventions are more invasive, and joint degeneration has begun. To improve screening and early diagnosis methods for DDH, the pathobiological mechanisms associated with this disorder must be further characterized. Therefore, the programmatic research plan for this dissertation investigated molecular biology, mechanobiology, biomechanics, and cell- and tissue-based disease mechanisms associated with DDH during its development and progression to secondary hip osteoarthritis. The primary objective for this body of work was to comprehensively characterize these stages of DDH in order to elucidate mechanistic biomarkers for diagnosis, staging, and treatment monitoring as well as targets for novel prevention and treatment strategies.

The first experiment in the dissertation research focused on clinically relevant biomechanics of the hip in order to characterize the relevant contributions from key soft tissue structures in maintaining hip stability. The data from this experiment indicated that the ligamentum teres of the femoral head and the acetabular labrum each play important and unique roles in hip joint stability. The ligamentum teres primarily supported anterior stability during increased hip flexion while the labrum primarily supported lateral stability during hip flexion and abduction.

The second set of experiments focused on the molecular biology of key intra-articular tissues of the hip by analyzing mechanistic metabolic responses in cell and tissue culture. First, metabolic responses related to hip osteoarthritis were characterized and analyzed for trends with histopathology severity scoring. The results indicated that each tissue type has a unique metabolic profile. Interestingly, the acetabular labrum was associated with robust inflammatory, degradative, immune cell recruitment, and anabolic responses during early degeneration followed by a pronounced anti-degradative response in late degeneration. Subsequent experiments used cells from canine DDH tissues to investigate mechanobiology-related responses prior to the onset of hip osteoarthritis. The results indicated that the ligamentum teres is a primary producer of inflammatory and immune cell recruitment proteins with labral cells also producing significant amounts of these proteins in response to supraphysiologic tensile loading. Interestingly, physiologic loading of dysplastic cells normalized their metabolic

response profiles to match those of healthy hips, indicating that dysplastic metabolic responses can be reversible in earlier stages of disease.

The final set of experiments for the dissertation focused on analysis of serum and urine from age-matched healthy individuals and those with symptomatic DDH prior to degeneration in order to elucidate mechanistic biomarker panels for differentiating hip status prior to the development of osteoarthritis. Patients with DDH prior to degeneration had significantly different serum and urine biomarker profiles compared to healthy-hip controls and patients with hip osteoarthritis. The primary differences involved increases in inflammatory biomarkers and decreases in bone metabolism, degradation, and anabolism biomarkers. When these proteins were combined into panels, there was excellent discriminatory capability for multiple serum and urine protein panels.

Taken together, this series of experiments provides novel molecular biology, mechanobiology, biomechanics, and cell- and tissue-based disease mechanisms data for characterizing DDH during its development and progression to secondary hip osteoarthritis. These data suggest that soft tissue structures in the hip, including the ligamentum teres and acetabular labrum, play key biomechanical and biologic roles in maintaining joint homeostasis and responding to the pathomechanisms involved in the development and progression of DDH. The experiments comprising this dissertation provide the foundation for additional translational and clinical studies designed to comprehensively characterize DDH development and progression and elucidate mechanistic biomarkers that can

consistently identify individuals with hip dysplasia at its earliest stages such that progression to hip osteoarthritis can be prevented.

CHAPTER 1:

INTRODUCTION

The hip is the diarthrodial joint that functionally links the lower extremities to the axial skeleton and allows for a wide range of movements.[1] The main structures within the hip are the articular cartilage[2–6], ligamentum teres[7–11], and the acetabular labrum[12–15]. **(Figure 1-1[16])** The hip joint is enclosed in a capsule that is lined by synovium.[17] The synovium secretes and filters the components of synovial fluid, which lubricates the joint and provides nutrients and metabolites that maintain joint homeostasis.[18–22] The articular cartilage covers the acetabulum, the cup portion of the joint, and femoral head, the ball portion of the joint, that are in contact with one another throughout movement.[23–25] The ligamentum teres is the ligament that connects the femoral head to the acetabulum.[7,26] The ligamentum teres is innervated and vascularized, playing a key role in providing blood supply to the femoral head during early development and contributing to hip joint stability later in life.[27–35] The acetabular labrum is a fibrocartilage structure that lines the rim of the acetabulum, creating a suction seal for the femoral head and also contributing to joint congruity and stability throughout movement.[36–42]

Developmental dysplasia of the hip (DDH) has been described as a complex multifactorial disorder that is influenced by genetics, epigenetics, birth and developmental factors, body habitus, and activities. DDH is anatomically described as a shallowing of the acetabulum such that the femoral head is not properly seated.[43,44] **(Figure 1-2)** When the joint is dysplastic, the hip becomes unstable

and can subluxate, or even luxate (dislocate), during activities of daily living.[45,46] According to the International Hip Dysplasia Institute[47], it has been reported that up to 1 in 100 infants are treated for DDH and 1 in 500 infants are born with a completely dislocated hip joint. DDH begins to develop around the time of birth and can be present in infants with no obvious abnormalities except for slight hip instability that can only be determined through clinical evaluation by a trained individual.[48–51] When DDH is diagnosed in infancy, treatment success is over 80% using non-invasive interventions such as a Pavlik harness or bracing **(Figure 2-11 & 12)**.[52,53]

When DDH goes undiagnosed in childhood, it most commonly presents as feelings of hip instability during activities, hip pain, and/or performance limitations for individuals in adolescence or early adulthood. DDH-related pain often originates from joint degeneration and associated inflammation, supraphysiologic loading on the supporting soft tissue structures, and associated strain on the surrounding musculature.[54–58] If these pathomechanisms are not appropriately addressed, DDH commonly progresses to early-onset hip osteoarthritis (OA).[59] Strikingly, adult DDH is reported to be 9 times more common than infantile DDH, and late detection of DDH, typically defined at >6-months of age[60], is associated with increased failure rates with conservative treatment. Consequently, invasive and costly surgical interventions may be required to address symptoms and preserve joint health and function.[61–63]

Secondary hip osteoarthritis (OA) is whole-joint degenerative disease that results from a known primary pathology, like DDH.[64,65] Patients with DDH and

symptomatic secondary hip OA often require hip replacement prior to 40 years of age.[66] DDH has been reported to be the primary cause of more than 25% of total hip arthroplasty (THA) performed in patients prior to the age of 40.[67] When a THA is performed prior to the age of 40, more than 17% require revision THA within 10 years, primarily due to mechanical fatigue of implants placed in this much younger and active patient population compared to the less active and older average THA population.[68] This 10-year revision rate for young patients is 3 times that of the average THA population, which is only 5.7%.[69]

Based on this preventable progression to secondary hip OA it is important to improve screening methods for early and accurate diagnosis of DDH to improve the employment of non-invasive and successful intervention. One key to achieving this goal is to elucidate the mechanisms by which DDH progresses to secondary hip OA in order to develop and validate targeted diagnostic, prognostic, preventative, and therapeutic strategies to aid clinicians in making more informed screening and treatment plans. While current treatment options are successful these improvements in planning will improve patient outcomes.

Analyzing protein biomarkers from body fluids provide a promising method for achieving improved screening. All joint tissues respond to biologic and biomechanical cues by synthesizing proteins to mitigate insults and injuries and restore homeostasis.[70–74] If these metabolic responses are insufficient or imbalanced and the damage is severe enough, irreversible degeneration ensues.[75–77] Consequently, many proteins from various metabolic response pathways are initially released from joint tissue into the synovial fluid.[78] Some

proteins from the synovium enter the blood stream and are eventually filtered through the kidneys to be excreted in urine.[79–81] Therefore, synovial fluid, blood and urine serve as potential sources of biomarkers of joint pathology, including DDH.[82–85]

The research in this dissertation will first utilize biomechanics to understand how the acetabular labrum and ligamentum teres provide stability to the hip through full range of motion. Biomarkers will then be utilized to understand how individual tissue production of proteins are altered by the pathology of osteoarthritis. Biomarker production is further utilized in investigating the acetabular labrum's metabolic trends to the histological scoring to understand the alteration in biomarker production as the tissue degenerates further. Biomarkers will then be investigated in a mechanobiology setting where dysplastic and healthy canine cells will be placed under altered biaxial stretching. This will facilitate understanding of the pathological state of dysplasia and how biaxial stretching affects tissues in the joint environment. Finally, biomarkers will be utilized in systemic human fluids. This fluid analysis will serve to better understand how biomarker production is altered in patients with hip osteoarthritis secondary to developmental dysplasia of the hip compared to individuals with developmental dysplasia of the hip prior to hip osteoarthritis. These fluid biomarkers will also be utilized in panels to investigate the viability of the biomarkers to differentiate individuals with developmental dysplasia of the hip prior to degeneration, while it is still treatable, to age matched healthy controls. The use of analyzing these biomarkers in these settings will allow for potential novel target treatment and

diagnostic options to reduce development of secondary hip osteoarthritis in those with developmental dysplasia of the hip.

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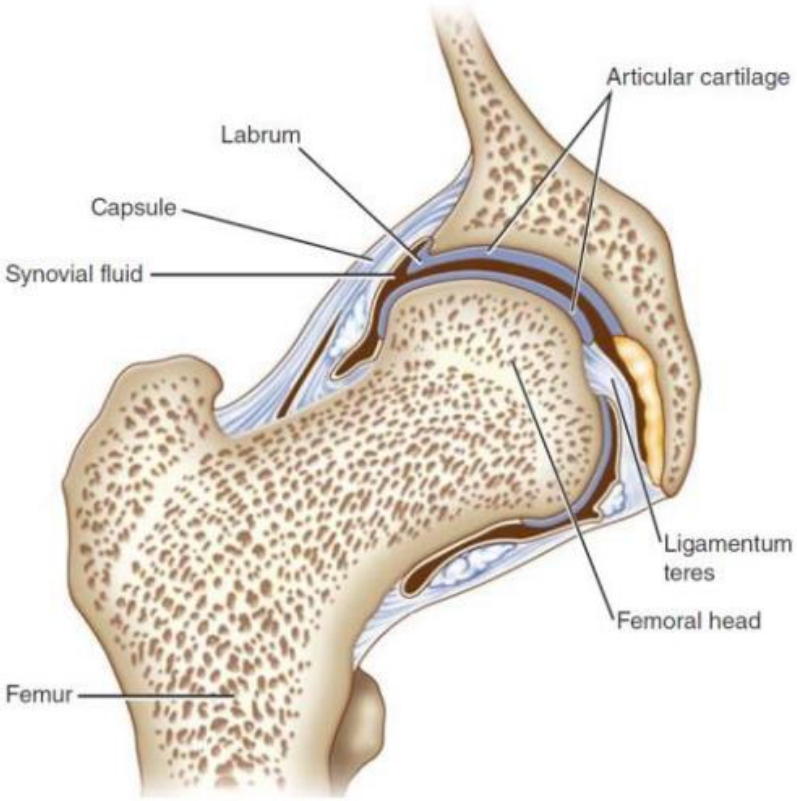

Figure 1-1: Hip joint anatomy. (FW Gwathmey, Patient's Guide to the Hip)

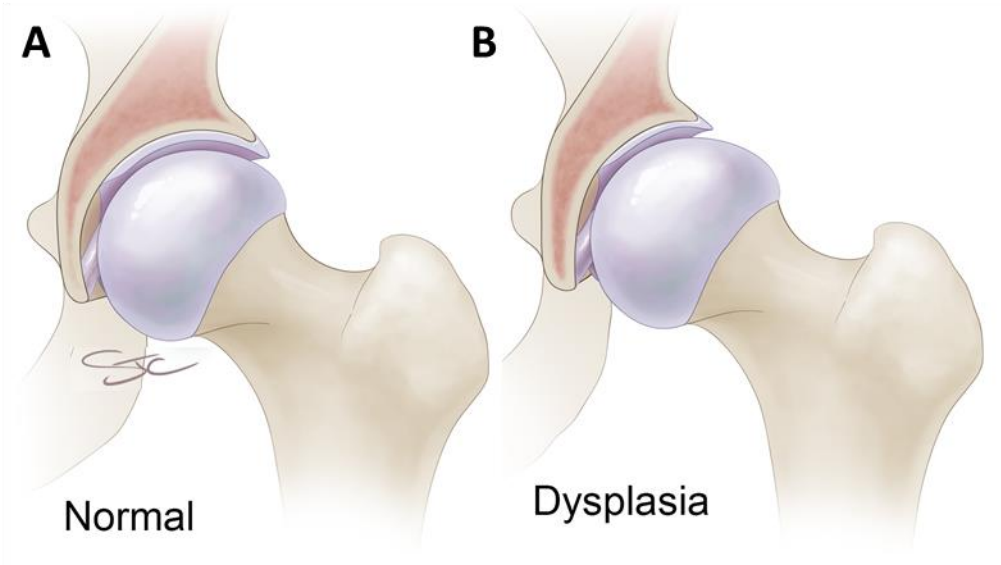

Figure 1-2: A.) Healthy hip with normal conformation, B.) Dysplastic hip with a shallowing of the acetabulum.

CHAPTER 2:

LITERATURE REVIEW

1. The Hip Joint and Osteoarthritis

1. a: The Hip Joint

The hip joint is a spheroidal joint[1,2] composed of two boney components and several soft tissue structures that are all essential to joint health and function.[3–7] The cup-shaped portion of the joint is the acetabulum, which forms by fusion of the triradiate projections of the ilium, pubis, and ischium [8,9] This structure bares the force of the lower extremity through range of motion and the acetabular depth is dependent on biomechanical cues that typically establish depth by the 8th to 9th year of age.[10,11] The second boney component of the hip joint is the ball-shaped portion of the proximal femur referred to as the femoral head. The proximal femur develops longitudinally from the epiphyseal growth plate which typically becomes fully developed, and fused, by the 19th year in females and the 21st year in males.[12,13] The angle between the femoral shaft and the femoral head is also critical to hip joint function because it can affect the force distribution across the joint, and the angle is determined by biomechanical forces during development.[14,15] Together, the functional development of the bone components of the hip joint are governed by genetics and epigenetics in combination with static and dynamic loading, which arise from joint morphology and movement patterns of daily life.[16,17]

The main soft tissue structures comprising the hip are articular cartilage,[18–22] acetabular labrum,[23–26] ligamentum teres,[27–31] and synovium.[32] The articular cartilage covers the apposing surfaces of the acetabulum and femoral head, providing a low-friction articulation for movement and allowing for dissipation of force during loading of the joint.[33–38] The articular cartilage is composed of the distinct superficial, middle, deep, and calcified zones with the type II collagen, aggrecan, and water as the primary extracellular matrix components in healthy tissues. The superficial zone is approximately 10-20% of the cartilage thickness and protects the deeper zones from shear stresses with the collagen being oriented in a parallel fashion.[39–41] The middle zone is approximately 40-60% of the cartilage thickness which provides compressive resistance.[41] The deep zone is approximately 30% of the cartilage thickness which provides even greater compressive resistance with the collagen being oriented in a perpendicular fashion.[41]

The acetabular labrum is a fibrocartilage structure which lines the rim of the acetabulum and adds to the stability of the joint by increasing acetabular depth and joint congruity.[42,43] The labrum also creates a fluid seal and negative pressure within the joint that reduce distraction of the femoral head.[43–51] The acetabular labrum consists of type I and II collagen with a majority being type I.[45] The articular surface of the labrum must maintain a smooth transition from labral fibrocartilage to acetabular hyaline cartilage.[43] This transition zone has collagen fibers that attach perpendicularly in the posterior portion of the joint while they attach parallel

in the anterior portion of the joint.[52] The non-articular side of the labrum directly attaches to acetabular bone where it is also innervated and vascularized.[26,43]

The ligamentum teres is the ligament which connects the femoral head to the acetabulum and is primarily described as providing nutrients to the femoral head through its vascularity during development.[27,53–56] This ligament is also described as providing secondary stabilization to the joint to prevent dislocation, especially at more extreme ranges of motion.[28,57] This ligament has also often been described as distributing synovial fluid throughout the joint by acting as a “windshield wiper” to spread the fluid with movement of the joint.[27,58] The ligament is described as the combination of three bundles, which are defined as anterior, posterior, and medial.[59] The ligamentum teres is predominately composed of type I, III, and IV collagen fibers along with vascularity and nerve bundles.[31,55]

Finally, the synovium is the tissue type that encapsulates the diarthrodial joint and produces and filters components of the synovial fluid, as well as play a major role in joint stability.[60–63] The synovium produces lubricin and hyaluronic acid to maintain the fluid volume and composition of the synovial fluid.[64] The synovium is composed of the intima and subintima with the outer layer being composed predominantly by type I collagen and is relatively hypocellular.[65] The inner layer is highly cellularized by type A and type B synoviocytes with the type B being the dominant cell type in healthy synovium.[65]

1. b: Hip Osteoarthritis

Hip osteoarthritis (OA) is a degenerative whole-joint disease that is associated with causes including aging[66], obesity[67], genetic mutations[68], developmental disorders[69], trauma[70,71], and overuse[72]. Hip OA is associated with wearing of the articular cartilage surfaces, subchondral bone alterations, and soft tissue inflammation and degeneration, leading to hip pain and dysfunction.[51–55] While primary hip OA, from an idiopathic, genetic, or aging origin, is still accepted as the primary pathway, secondary hip OA, resulting from a known disorder, insult, or injury, is becoming more widely recognized.[73] A prominent cause of secondary hip OA is developmental dysplasia of the hip (DDH).[74,75] Importantly, DDH can be effectively treated when detected prior to the onset of degenerative joint disease such that secondary hip OA can be delayed or even prevented.[76]

2. Hip Dysplasia

Developmental dysplasia of the hip (DDH) is a complex multifactorial disorder that can be difficult to diagnose and effectively treat.[77,78] DDH encompasses a spectrum from asymptomatic shallowing of the acetabulum to complete dislocation of the femoral head.[79,80] If the disorder is undiagnosed in infancy, the prevention and treatment options lose effectiveness and risk for developing secondary osteoarthritis increases with age at initial diagnosis.[81,82]

2. a: General Overview

DDH was first described by Hippocrates (around 400 BC) as hip dislocation in infants, and he believed that the joint dislocation occurred due to a potential

traumatic impact or compression to the womb.[83] It was not until the 1500s when Ambroise Paré began to describe hip dislocation in young patients in more detail, and proposed that the proper relationship of the femoral head and acetabulum could be restored without surgery through physical reduction by a physician.[84] In the early 1800s, Baron Depuytren described hip dislocation in young patients further, hypothesizing that positioning of the fetus could influence joint development and lead to shallowness of the acetabulum causing hip dislocation.[85] Depuytren termed this condition as congenital dislocation of the hip (CDH) and indicated that it may occur more frequently than originally believed.[86] Depuytren reported that a majority of the patients presenting with this deformity were female, which holds true to this day.[87] Late in the 1800s, A.M. Phelps, a surgeon, provided data to support Depuytren's theories by using anatomic specimens to show hip dislocations did happen *in utero* or during birth.[83]

The term CDH was used until 1989 when there was a report suggesting that it was an unsatisfactory term based on an emerging spectrum of associated hip pathology that did not always result in dislocation.[88] The same report also claimed that dislocation, and other manifestations of the disorder, frequently happened postnatally, suggesting that it may not be a congenital pathology.[88] The report suggested the use of the term "Developmental Displacement of the Hip" to describe the dynamic disorder that embraced the variants of timing and pathology.[88] The American Academy of Orthopaedic Surgeons adopted the term, Developmental Dysplasia of the Hip (DDH), in 2000.[89] This term was also accepted by the Japanese Paediatric Orthopaedic Association in 2004.[90]

2. b: Epidemiology

DDH presents as hip instability in estimated at 1 in 10 births, dislocated hips in approximately 1 in 500 children born, and approximately 1 in 100 children are treated for DDH.[91,92] The lowest incidence for DDH is reported in Africa at a rate of 0.06 per 1000 births, while the highest incidence is reported for Native Americans at rate of 76.1 per 1000 births.[93] DDH affects females (approximately 19 out of 1000) at a higher rate than males (approximately 4 out of 1000) with approximately 80% of cases being female.[93–95] It has been reported that 95-98% of DDH cases in infants are reversible with early diagnosis and treatment.[96]

2. c: Causes

Etiology of DDH is multifactorial with numerous factors contributing to its various clinical manifestations. Breech-position birth is associated with an increased odds ratio of 5.7 (95% CI 4.4-7.4) for DDH.[97] This mispositioning *in utero* alters fetal biomechanics and hip joint development, which is suspected to result in underdevelopment of the acetabulum and increased risk for DDH.[98] Infants who suffer from oligohydramnios (reduced amniotic fluid) have an increased odds ratio of 3.9 (95% CI 2.1-7.3) for DDH.[99] The smaller womb volume is proposed to detrimentally affect *in utero* hip biomechanics and acetabular development.[98] Infants with a family history of DDH have an increased odds ratio of 4.8 (95% CI 2.8-8.2).[97] Epigenetics factors such as increased weight, postnatal trauma, hormonal imbalances, and neuromuscular immaturity have been described to

differentially express genes which may affect the pathomechanisms of DDH.[100–102] There is also the postnatal positioning of the hip, breech positioning, that has been shown to increase the risk of DDH by placing increased sustained force on the hamstring that may contribute to subsequent hip instability.[103–105] Swaddling an infant into a straight leg position contributes to DDH with an increased odds ratio of 8.65 (95% CI 2.23-33.57) due to the forced extension and adduction being placed on the hip joint.[106]

2. *d: Early Diagnosis*

Diagnosis of DDH begins with the physical examination shortly after birth.[107,108] When diagnosis from physical examination needs further confirmation, or a patient is considered high risk based on several known risk factors, an ultrasound can be used to further investigate the joint morphology[109]. Early and accurate diagnosis is imperative to timely administration of interventions to allow for maximum potential of resolution, and no subsequent intervention, of DDH.[110–112] Early intervention allows for prevention of subsequent secondary hip OA from DDH when the joint morphology is corrected.[113]

i. General Examination

Shortly after birth, or at the initial well-baby checkup within the first three months of life, a trained individual assesses the infant for risk factors of DDH and examines the infant.[114,115] Risk factors considered at this time point include breech birth, oligohydramnios, being an infant from a multiple birth, and family history of

DDH.[97,116–118] Physical examination findings suggestive of DDH include asymmetrical buttock and thigh skin creases and relative asymmetry in femoral length at 90° of hip flexion, indicating abnormal hip positioning.[119–122]

Physical examination maneuvers include two tests for hip laxity and/or subluxation-luxation indicative of DDH.**(Figure 2-1**[123]**)** The Barlow test places the hip at 90° flexion and the examiner places a hand on the lateral thigh to exert posterior pressure on the hip while the lower extremity is adducted, assessing for posterior hip subluxation-luxation felt as a “click” or “slip”.[124] The Ortolani test places the hip at 90° flexion and the examiner places a hand on the lateral thigh below the femoral head while performing slow abduction of the lower extremity to feel for a “clunk” in the hip joint associated with reduction of a subluxated-luxated femoral head into the acetabulum.[125,126] The Ortolani test is most accurate prior to six weeks of age but can be confused with ligament snapping in a stable joint in older infants.[127] While these provocative maneuvers are key tools for screening and early diagnosis, they require an examiner with expertise in the maneuvers. These examinations are subjective and a 15-year observational cohort study, with a large sample size of 70,071, reported 16.7% sensitivity, 99.8% specificity, 3.5% positive predictive value, and a 100% negative predictive value.[128]

ii. Ultrasound

Ultrasonography of the hip can provide a more definitive diagnosis of DDH in infants as it allows for subjective and objective assessments of the incompletely ossified joint structures without ionizing radiation.[129] With ultrasonographic

examination, metrics for the boney structures are used to determine morphology of the hip joint and acetabular coverage of the femoral head.[130] The primary measurement used for ultrasonographic diagnosis of DDH is the alpha angle formed at the convexity of the ilium from the center of the labrum and where the ilium meets the acetabulum.[131]**(Figure 2-2**[132]**)** An alpha angle of ≥60° is considered normal while an angle <60° may be indicative of hip dysplasia.[133] Percentage of acetabular coverage can also be calculated by determining the ratio of the distance of the acetabular depth, from the acetabular roof to the iliac line, over the diameter of the femoral head.[134]**(Figure 2-3**[134]**)** A sufficient coverage is ≥50% while <50% is considered dysplastic.[134–136] The ultrasound examination is typically conducted around 3 months of age, following the indication of instability of the hip or a family history, and requires highly-trained individuals for accurate diagnosis and subjective assessment of acetabular morphology.[137] A 15-year prospective study reported that ultrasonographic assessment for DDH was associated with 77% sensitivity, 99.84% specificity, and 49% positive predictive value.[138]

2. *e: Delayed Diagnosis*

Missed or misdiagnosis of DDH in infancy often leads to a delay in diagnosis into adolescence or young adulthood.[139] When diagnosis is delayed, it is often based on patients' symptomatic presentation of hip pain and abnormal gait which requires an in-depth physical examination to understand the origin of these symptoms.[140,141] In these patients, the diagnostic assessment should include physical examination, radiographs, and potentially advanced imaging.[142–145]

Comprehensive assessment is essential in order to determine a definitive diagnosis, and subsequent stage for the severity of dysplasia and joint degeneration.[146]

i. General Examination

When patients present for treatment of hip dysplasia in adolescence or adulthood, it is often because of hip pain and dysfunction.[147] A complete physical examination helps to differentiate DDH from other disorders that cause similar symptoms and sources of dysfunction.[143] In these older DDH patients, pain is often present in the front of the groin and this pain is associated with labral tears, cartilage damage, and hip flexor overuse from hip instability.[140] These individuals may also complain of mechanical symptoms including joint catching, popping, and locking.[140] Hip dysfunction may manifest as activity limitations, or a limp, and may be related to joint pain, stiffness, leg length discrepancy, or compensatory change in gait to reduce pain or mechanical symptoms.[140] While these signs are not each individually specific to DDH, taken together, they help in diagnosis of the underlying condition.[143]

The physical examination includes assessment of standing pelvic tilt, described as the anterior or posterior angle in relation to a vertical axis, and leg length.[148,149] Increased anterior pelvic tilt to compensate for the lack of acetabular coverage is common in DDH patients.[150] During the walking portion of the physical examination, a limp with the Tredelenburg sign of a hip “dropping” to the affected side can be suggestive of DDH.[151] The examiner should also perform a complete

range of motion examination. The first maneuver in this exam includes both internal and external rotation to passive range of motion past 45° to check for a clicking sound as indication of a labral pathology.[152] The second maneuver is performed with the patient laying on their side with abduction of 30-45° of the upward facing extremity and externally rotating the hip with anterior pressure placed by the examiner assess for instability of the hip.[152] The third maneuver is the FABER test, performed with the patient supine flexing the hip to 90° while simultaneously abducting and externally rotating the hip. Pain at the end range of this maneuver is indicative of labral pathology or DDH.[153] **(Figure 2-4**[154]**)** The fourth maneuver is the FADIR test, performed with the patient supine flexing the hip to 90° and simultaneously adducting and internally rotating the hip. Pain and clicking at the end range of FADIR test is indicative of intra-articular hip pathology or DDH.[155] **(Figure 2-4**[156]**)**

ii. Radiographic Assessment

Anterior-posterior (AP), frog leg, and the false profile radiographic views allow for assessment of femoral head sphericity and symmetry, acetabular coverage, and relationship between the femoral head and acetabulum.[157] The most common radiographic measurement for determination of the presence of DDH is the center-edge angle which is captured from the AP view in a standing a supine position.[158] The center-edge angle is the angle formed by a line connecting the center of the femoral head perpendicular to the transverse axis of the pelvis and the line connecting the center of the femoral head and the uppermost lateral

point of the acetabular sclerosis.[159,160]**(Figure 2-5**[161]**)** An angle of ≥25° is considered normal acetabular coverage of the femoral head, <25° and ≥20° as borderline dysplasia, and <20° as under-coverage and presence of DDH.[162] This measurement is only considered reliable when the patient being radiographed is >5 years of age.[162,163] The frog leg view is obtained with the hip joint at flexion of 30° and abduction of 45° allowing for the same measure to be collected while also determining if the femoral head is reducible into the acetabulum.[164] The false profile view is obtained with the pelvis rotated 65° in relation to the radiographic film with the side being analyzed in contact and parallel to the cassette.[165]**(Figure 2-6**[165]**)** This view allows for the anterior center-edge angle to be assessed by measuring the angle between the vertical line through the femoral head and the most anterior point of the acetabular sourcil.[166]**(Figure 2-7**[165]**)** An anterior center-edge angle <25° indicates under coverage of the acetabulum over the femoral head and DDH.[159] The Tönnis angle is measured from the AP view to determine the acetabular inclination by first drawing a horizontal line between the acetabular teardrops along with a parallel line that runs through the most inferior point of the sclerotic acetabular sourcil.[165] A third line from the inferior sourcil to the lateral sourcil is determined and the angle at the inferior sourcil is the Tönnis angle with a measurement of >10° indicative of DDH.[165]**(Figure 2-8**[165]**)** The final measurement utilized is the femoral head extrusion index taken from an AP view.[167] This measurement is determined by the distance from the medial point for the femoral head to the lateral acetabular rim over the total diameter of the femoral

head multiplied by 100 to obtain a percentage with a >25% indicating DDH.[167,168]**(Figure 2-9**[169]**)**

The Crowe classification system is a radiographic assessment to determine severity of DDH.[170] The Crowe classification is performed using the AP radiograph of the full pelvis to determine the height of the femoral head in relation to the height of the pelvis.[171] First, a horizontal line is placed between the inferior portions of the inferior acetabular wall. Next, the distance is measured between this horizontal line and the inferior portion of the femoral head-neck junction.[171] This distance is placed over a fifth of the height of the pelvis which is measured from the anterior point of the iliac crest and inferior point of the ischial tuberosity, and this total value is the subluxation percentage.[172]**(Figure 2-10**[173]**)** Grade 1 is indicated by <50%, grade 2 by 50-75%, grade 3 by 75-100%, and grade 4 by >100%.[174] Higher Crowe classification grades have been reported to be associated with higher complication rates after surgical intervention due to the unfavorable joint morphology and stability.[172,174–177]

Radiographs are also utilized in determining the presence, and degree, of hip OA using the Tönnis classification system. The Tönnis system is based on a 4-point grading scale with 0 indicating no OA and 3 indicating severe OA.[178] The grade is determined by evaluating the width of the joint space, presence of subchondral cystic changes, density of the subchondral bone, and the sphericity of the femoral head.[178]**(Table 2-1**[179]**)**

iii. Advanced Imaging

While advanced imaging, such as magnetic resonance imaging (MRI) and computed tomography (CT), can provide a wealth of information for treatment of DDH, it is not used for routine diagnosis or staging purposes based on related necessity and associated costs.[142,180,181] When needed, MRI is used preoperatively for its ability to image soft tissues and cartilage to determine the severity, location, and size of cartilage and labral defects along with associated ligament, tendon, and muscle pathology.[182,183] MRI can also be useful following casting or after joint-preservation surgeries in order to ensure the femoral head has been reduced fully, the joint is not placed at an improper abduction angle that may increase likelihood for avascular necrosis, and soft tissue structures are healing appropriately.[184–187] MRI imaging is routinely selected over CT when advanced imaging is completed on an infant because the MRI does not produce ionizing radiation.[188]

CT is utilized in a similar manner to MRI but is better for imaging bone than soft tissues and it delivers ionizing radiation for imaging.[189] CT allows for imaging through casting material without image deterioration, which is important for imaging infants in casts or splints.[190] CT also requires shorter acquisition time and more readily allows for imaging with surgical implants compared with MRI with standard protocols.[191] Both CT and MRI can be utilized for assessing femoral torsion and acetabular version, but the MRI does require the inclusion of the femoral condyles for femoral torsion.[190] Both CT and MRI allow for images to be reconstructed into 3D renderings, which can be helpful for assessing all related

pathology and surgical planning for reorientation procedures, such as the periacetabular osteotomy (PAO).[192–194]

2. f: Treatments

Treatment options for DDH are dependent on the time of diagnosis, degree of dysplasia, and severity of OA in conjunction with patient demographics, medical comorbidities, activities, and expectations.[195–199] Currently, treatment options are categorized as preventative, joint-preserving, and salvage procedures.[200] Preventative treatments focus on correction of DDH when the joint is still developing to address instability and improve acetabular depth to mitigate progression to secondary hip OA.[198] These preventative treatments are best implemented within the first 4 years of life such that early and accurate diagnosis is imperative.[198,199,201] Once a patient has passed the window for successful implementation of preventative techniques, joint-preserving surgical options are considered for treatment of symptomatic DDH prior to degenerative joint disease. The joint-preserving surgery most often utilized, PAO, involves reorientation of the acetabulum to improve joint stability, kinematics, function, and health. Proximal femoral rotational osteotomy (PFRO) may be performed in conjunction with PAO or as an isolated procedure to address abnormal femoral torsion when it is determined to significantly affect to joint stability/stress.[202] The goal is to reorient and normalize joint biomechanics to slow the rate of cartilage loss and associated joint degeneration in order to either prevent secondary OA or at least slow its development until salvage procedures can be performed at a more appropriate

age.[203] The most commonly performed and functionally successful salvage procedure is total hip arthroplasty (THA).[204] The decision of a joint-preserving or salvage procedure is complicated and takes into consideration the degree of dysplasia, cartilage degeneration, patient age, medical comorbidities, and the level of activity that is desired from the patients.[205–208] Increased severity of dysplasia and cartilage degeneration are associated with higher likelihood for negative outcomes following joint-preservation surgery.[209,210]

i. Preventative

When DDH is detected in the first six months of life, the treatment options are non-surgical and aim to prevent the progression of DDH.[211] If the hip is not dislocated and the DDH is not severe, the first option is the Pavlik harness.[199] The Pavlik harness is a soft splint which straps over the infant's shoulders and under the feet.[212]**(Figure 2-11**[213]**)** This option allows for the infant to move and keeps the hips in an ideal position to promote alignment and muscle recruitment to aid in proper development of the acetabulum.[214] The harness passively maintains the hip in flexion, abduction, and external rotation; while active forces are placed on the joint by the infant when it pushes its feet on the foot straps.[215–217] When the Pavlik harness is administered properly, the success rate for the correction of DDH is reported to be above 75%.[218] With the Pavlik harness, there is approximately a 10% complication rate of developing avascular necrosis (AVN) in the least severe DDH cases with an increased risk, up to 30%, in more severe cases.[219] Likewise,

femoral nerve palsy may occur, but less frequently, at 2-3% with a higher frequency associated with increased severity of DDH.[220]

When DDH is more severe and hips are dislocated, or the infant fails the Pavlik harness treatment, another preventative treatment option is the fixed-abduction brace.[221,222]**(Figure 2-12**[223]**)** As the name implies, the brace holds the hips in abduction of up to 60° and flexion up to 100°.[224] This treatment option has a success rate for correction reported up to 92% when bracing is utilized properly.[225] Femoral head AVN rates may be up to 13%.[226,227]

If bracing alone fails, or the infant is over six months of age and DDH has progressed, then closed reduction with subsequent bracing is utilized.[228] Closed reduction of the hip is a manual manipulation of the femoral head into correct position within the acetabulum. Bracing is then applied to maintain reduction of the hip.[229,230] Subsequent radiographic imaging is used to ensure correct reduction has been achieved.[231] The rate of successful correction following a closed reduction and bracing is approximately 70% with a significant increase in failure in patients >18 months of age.[232] The risk of avascular necrosis is approximately 10% with the highest risk associated with increased DDH grade.[232,233]

If the previous non-surgical treatments fail to correct the hip instability, or diagnosis occurs after eighteen months of age, then an open reduction with subsequent casting can be attempted.[234] Open reduction involves surgical exposure of the hip joint to remove any obstructions that may be preventing femoral head seating within the acetabulum, followed by capsulorrhaphy, acetabuloplasty, or a combination of these techniques.[235–237] The iliopsoas,

ligamentum teres, transverse ligament, and fibro-fatty tissues filling the acetabular fossa (pulvinar) are the common structures that are resected to improve reducibility.[238] The success rate after open reduction was reported to be approximately 78% while 19% of patients developed femoral head AVN.[239]

ii. Joint-Preserving

When dysplasia is severe, or uncorrected by previous treatment options, one or more osteotomies may be performed to change the length or alignment of the acetabulum and/or femur.[240] Osteotomies have the highest success rate in individuals younger than 35 years of age unless there is cartilage degeneration already present based on MRI or arthroscopy.[241–246] For DDH, the most common osteotomy performed prior to skeletal maturity and fusion of the triradiate cartilage is the Pemberton osteotomy. The Pemberton osteotomy is characterized by a redirection of the acetabular roof, hinged on the triradiate cartilage after an incomplete iliac osteotomy.[247]**(Figure 2-13**[248]**)** When the operation occurs prior to 4 years of age there was a 93.6% rate of excellent to good results at the 15-year follow-up, but these positive outcome rates decrease with increases in age at the time of procedure with 56.7% of patients 4-10 years of age having excellent to good outcomes.[249] A periacetabular osteotomy (PAO) is the common osteotomy administered following skeletal maturity.[250]**(Figure 2-14**[251]**)** PAO returns hip joint stress distribution to patterns that are within normal limits for healthy hips.[252–254] Studies have reported that PAOs have an 18-year survival rate of 74% (95% CI of 66-83%) with an average of 21% being converted to total hip arthroplasty (THA)

and the average conversion rate occurring at 9 years following the osteotomy.[255] These conversions commonly occur due to the progression of osteoarthritis and the presence of cartilage damage. The 30-year survivorship of PAOs is only 29% with >70% of PAOs being converted to THAs due to progressive osteoarthritis.[256] Reported major complications after PAO includes heterotopic ossification, acetabular migration, nonunion, and venous thromboembolism at a combined rate of 5.8%.[257]

The femoral osteotomy is administered to the proximal portion of the femur and utilized when there is a rotational deformity which increases the risk of redislocation.[238] This procedure is referred to as the varus de-rotational osteotomy (VDRO) because it reduces the varus deformity seen in the proximal femur of those with DDH and returns more native forces to the hip joint.[258]**(Figure 2-15**[248]**)** VDRO has been shown to have satisfactory results in 78.5% of cases with a 9.4% complication rate, of which 2.5% are femoral head AVN.[259,260]

iii. Salvage

When previous procedures fail or DDH has progressed to symptomatic degenerative joint disease, a surgical salvage procedure is indicated.[261] Total hip arthroplasty (THA) is the most common salvage procedure performed, and only in very rare cases would excision arthroplasty, or arthrodesis, be considered when a THA is not feasible due to advanced bone defects or severely compromised bone structures which would not allow for the implantation of the THA device.[262] THA in DDH patients is more complex than primary hip OA patients because of the

shallowness of the acetabulum, their younger age, previous surgical procedures performed, and discrepancies in leg length.[204,263–265] As such, THA in this patient population is associated with average failure rates of 16% at 10 years and 40% at 20 years.[266,267] Complications rates associated with THAs performed in those with DDH are reported to be up to 27% for intraoperative fractures along with a 26% aseptic loosening rate at 10 years.[268]

3. Pathobiology

As outlined above, current diagnostic approaches for DDH are largely subjective and require judgement from highly trained and experienced individuals. The subjective nature of current diagnostics results in relatively high rates of misdiagnosis, which can lead to missed opportunities for prevention, delayed treatment, and diminished outcomes. Objective and quantitative assessments of DDH to determine the pathomechanisms related to development and progression of DDH to secondary OA are needed. The primary objective for this body of work was to comprehensively characterize these stages of DDH to effectively elucidate alterations in joint biomechanics, mechanistic biomarkers for diagnosis, staging, and treatment monitoring as well as targets for novel prevention and treatment strategies.

3. a: Biomechanics

Hip biomechanics play key roles in development, progression, prevention, and treatment of DDH. As such, biomechanical analysis of the hip joint and the

function of the proposed stabilizing structures in contributing to joint kinematics in healthy and DDH hips is critical for characterizing mechanisms of disease. In particular, the translations of the femoral head are of importance in the context of DDH as they are described to be increased from the undercovering of the femoral head by the acetabulum.[269–271] The acetabular labrum is described to primarily provide lateral stability and protect against joint distraction under lateral loading of the hip at various flexion angles.[272] The ligamentum teres has been proposed to primarily provide anterior stability when the hip is placed in adduction, flexion, and external rotation movements.[273,274] Characterization of the specific contributions of these stabilizing soft tissue structures with respect to femoral head translations during clinically applicable movements is a critical step in elucidating pathomechanisms and targets for prevention and treatment.

3. b: Mechanobiology

The field of mechanobiology focuses on the effects of physical force on cells and tissues related to their development, differentiation, physiology, and metabolic responses in health and disease.[275] Musculoskeletal tissues including ligaments, tendons, meniscus, bone, and cartilage have been studied in the context of mechanobiology and shown to have unique responses to loading, but ligamentum teres and acetabular labrum have yet to be studied in this context.[276–280] Based on the importance of ligamentum teres and acetabular labrum in DDH[26,274], characterizing the mechanobiology of these two tissues and the resultant effects

the hip joint environment in DDH and its progression to secondary hip OA is a critical component of programmatic research in this area.

3. c: Tissue Metabolism

In orthopaedic research, there has been increased interest in the metabolism of specific tissue types in various stages of disease to elucidate regulatory pathways underlying specific pathomechanisms.[281–283] Characterizing the tissue-specific metabolism of ligamentum teres, acetabular labrum, synovium, and cartilage in healthy and diseased hips is a foundational step for determining mechanisms of disease in DDH and hip OA and delineating targets for early and accurate diagnosis, prevention, and treatment of hip pathology.[284,285] Determining trends and patterns for tissue-specific metabolic profiles with histopathologic severity grading provides a method for uncovering the pathways and processes that drive the characteristic clinical findings recognized in patients with DDH.[286–289]

3. d: Systemic Biomarkers

Biomarkers measured in serum, urine, and synovial fluid have been investigated as a potential method for disease screening, diagnosis, and staging for joint disorders.[290–293] Recently, protein biomarker profiles measured in the serum and urine of 5-month-old dogs were reported to be effective for predicting hip status as dysplastic or healthy at 2 years of age.[294,295] Based on the similarities between canine hip dysplasia and DDH in human patients[296,297], serum and urine protein biomarkers appear to have strong potential for addressing a critical unmet

need in orthopaedics. The first key step toward this goal involves the elucidation of disease mechanisms that define clinically relevant stages in the development and progression of DDH. The second key step toward this goal involves creating protein biomarker panels for differentiation of hip status prior to the progression of hip OA secondary to DDH. This would allow validation of clinically applicable and cost-efficient serum and/or urine biomarker panels for screening, diagnosis, and staging for DDH.[298–301]

4. Study Design

DDH and its progression to osteoarthritis is a multifactorial disease that can affect all structures of the joint. The current treatment options are dependent on the age of diagnosis and accurate diagnosis which is currently based on subjective measures that have suboptimal sensitivity. To improve screening and early diagnosis methods for DDH, the pathobiological mechanisms associated with this disorder must be further characterized. Therefore, the programmatic research plan for this dissertation investigated molecular biology, mechanobiology, biomechanics, and cell- and tissue-based disease mechanisms associated with DDH during its development and progression to secondary hip osteoarthritis. The overall goal for this research is to comprehensively characterize these stages of DDH to elucidate mechanistic biomarkers for diagnosis, staging, and treatment monitoring as well as targets for novel prevention and treatment strategies.

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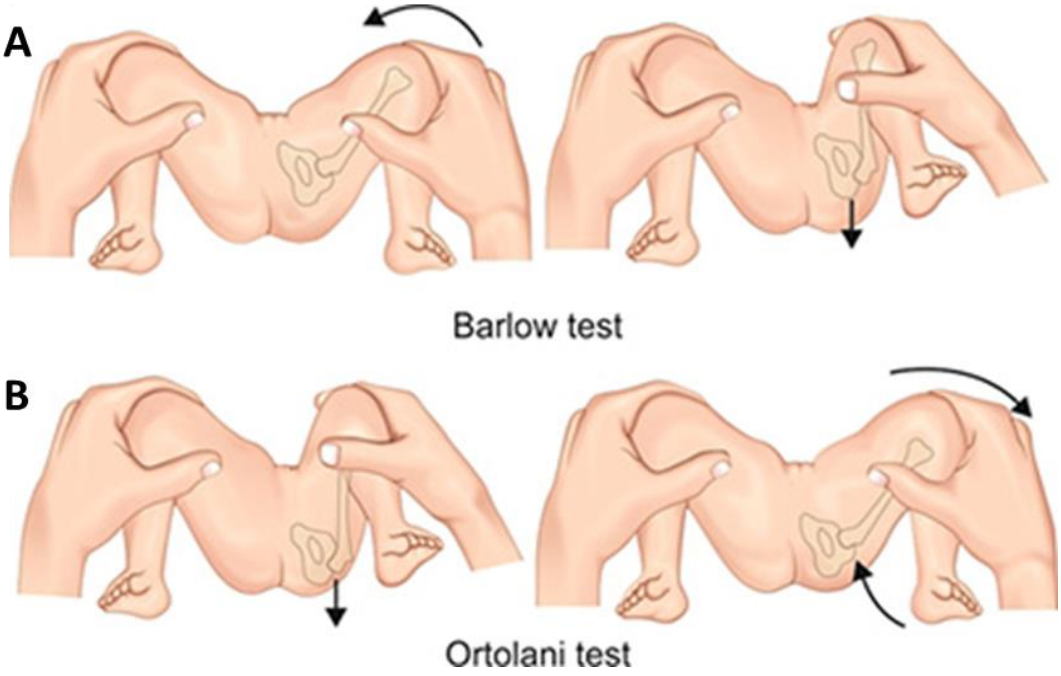

Figure 2-1: A.) Barlow test, B.) Ortolani test for developmental dysplasia of the hip in infants (MK Varshney, Essential Orthopedics: Principles & Practice, 2016)

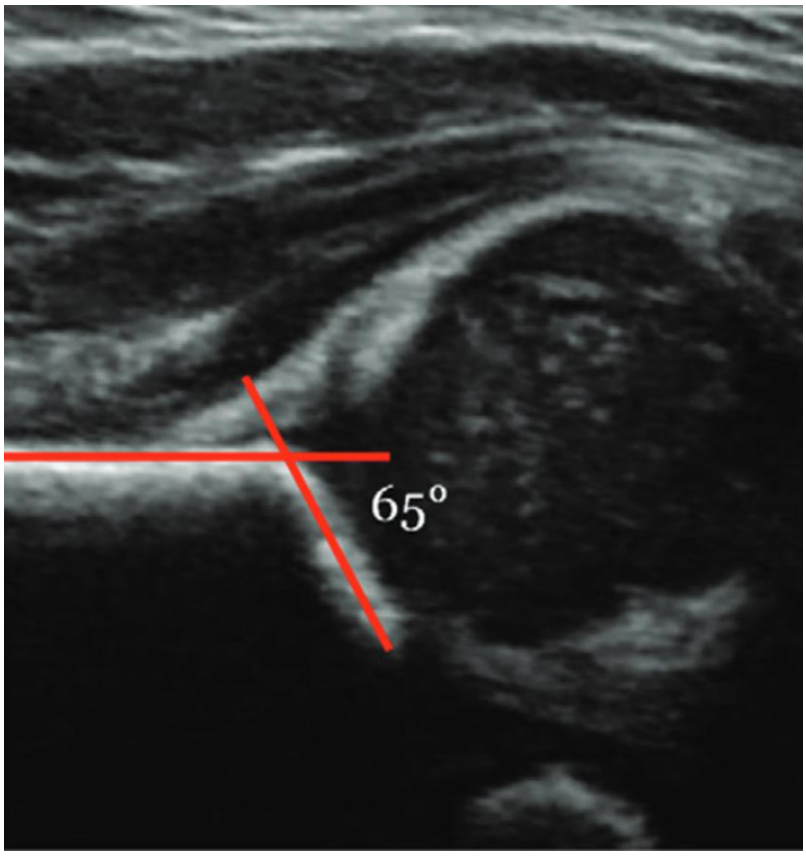

Figure 2-2: The alpha angle measurement of the hip joint from ultrasound examination (JL Jaremko, Potential for change in US Diagnosis of hip Dysplasia solely caused by changes in Probe Orientation: Patterns of Alpha-angle Variation Revealed by Using Three-dimensional US, 2014)

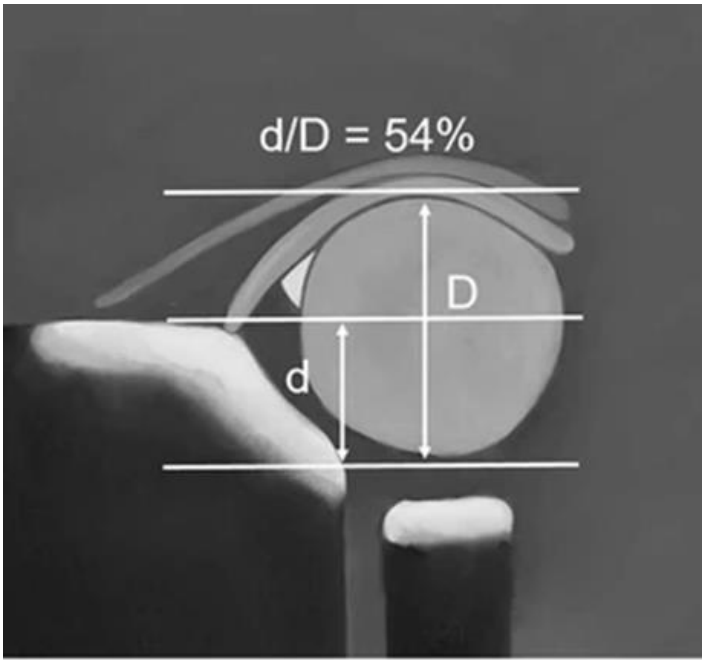

Figure 2-3: Schematic representation of the acetabular depth measurement with d representing the distance from the acetabular roof to the iliac line and D representing the diameter of the femoral head (H Harcke, Hip ultrasound for developmental dysplasia: the 50% rule, 2017)

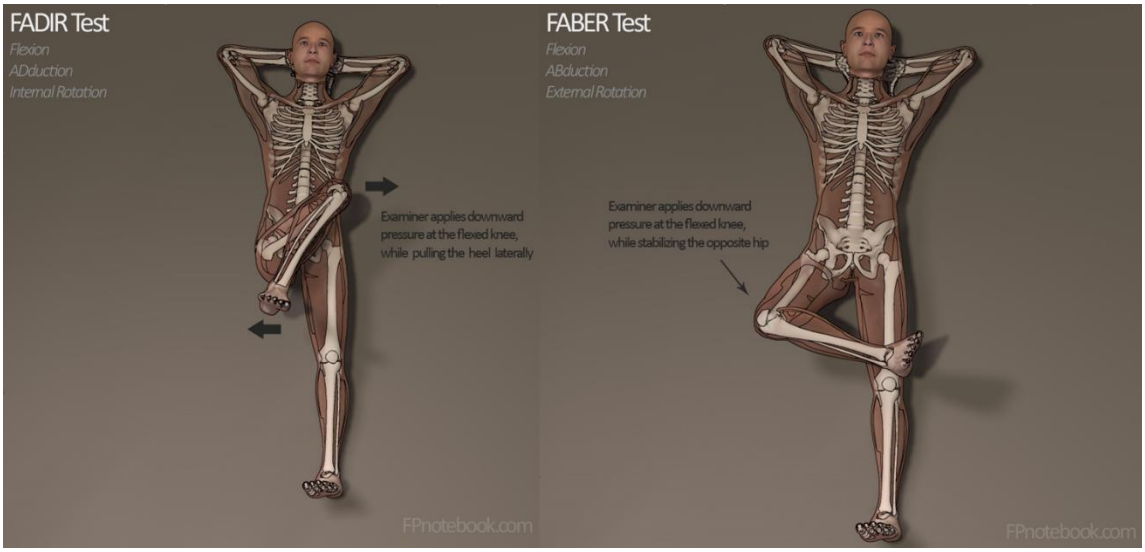

Figure 2-4: Representation of the FADIR and FABER clinical exams to test for hip pathology (S Moses, Family Practice Notebook, 2021)

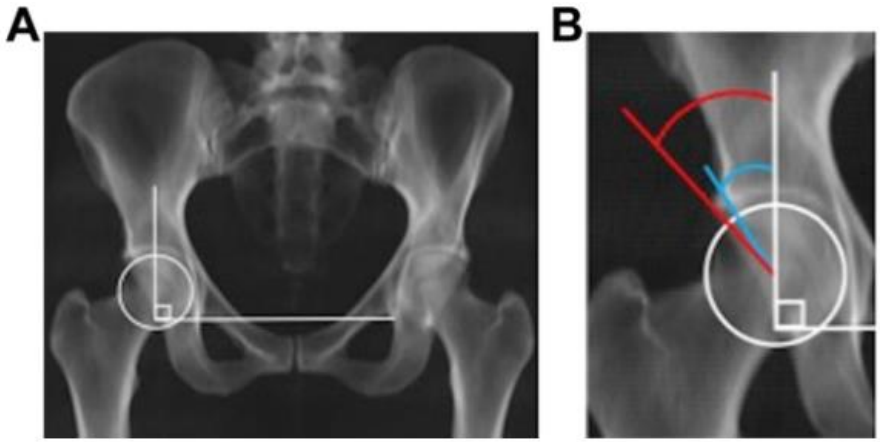

Figure 2-5: Representative measurement of the center edge angle. A.) The horizontal line of the pelvis and the connecting perpendicular line running through the femoral head. B.) Maginfied view of the femoral head with the measurement of the vertical line at the center of the femoral head to the lateral point of the acetabular sclerosis (JD Wylie, Relationship Between the Lateral Center-Edge Angle and 3-Dimensional Acetabular Coverage, 2017)

Figure 2-6: Representative positioning for a false-profile view of the right hip with the pelvis rotated 65° in relation to the cassette and the affected side3 parallel to the cassette. (JC Clohisy, A Systematic Approach to the Plain Radiographic Evaluation of the Young Adult Hip. 2008)

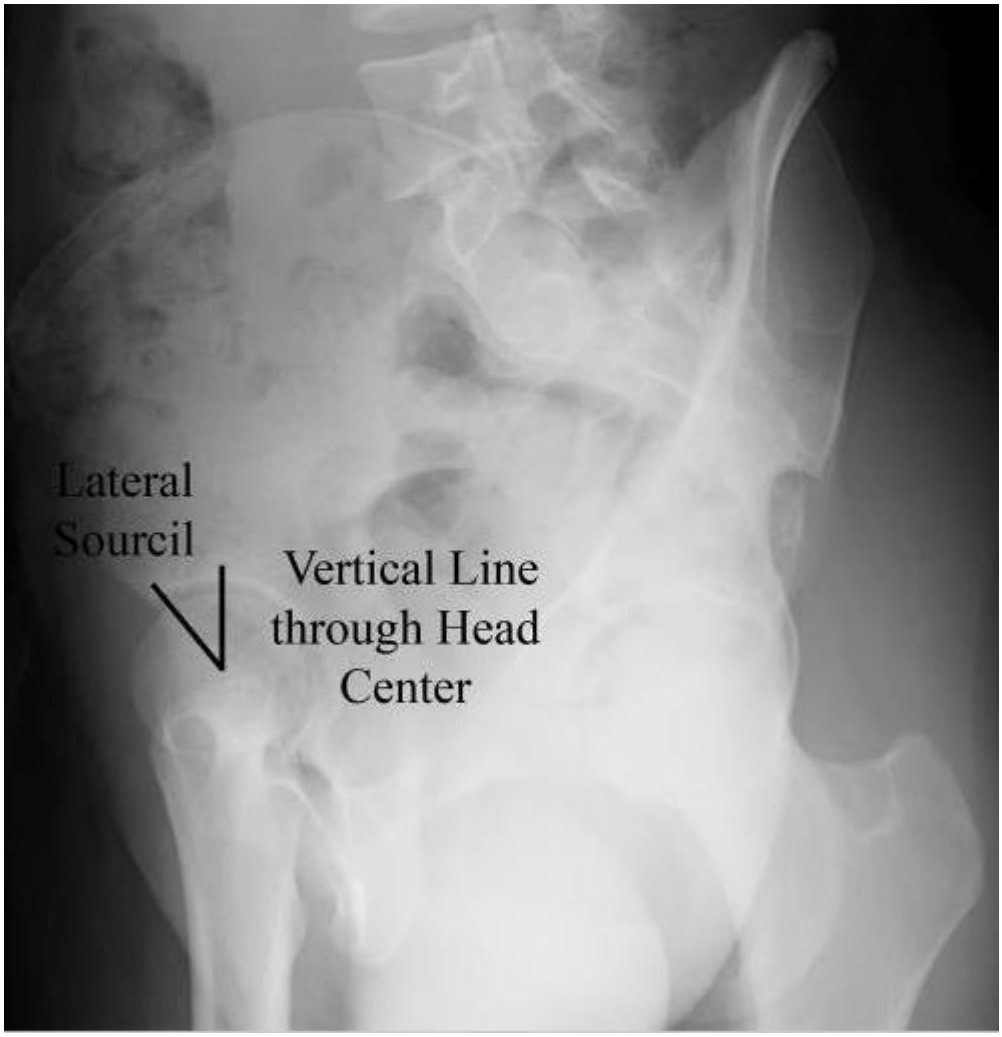

Figure 2-7: Representative technique for calculating the anterior center-edge angle on a false-profile radiograph. (JC Clohisy, A Systematic Approach to the Plain Radiographic Evaluation of the Young Adult Hip. 2008)

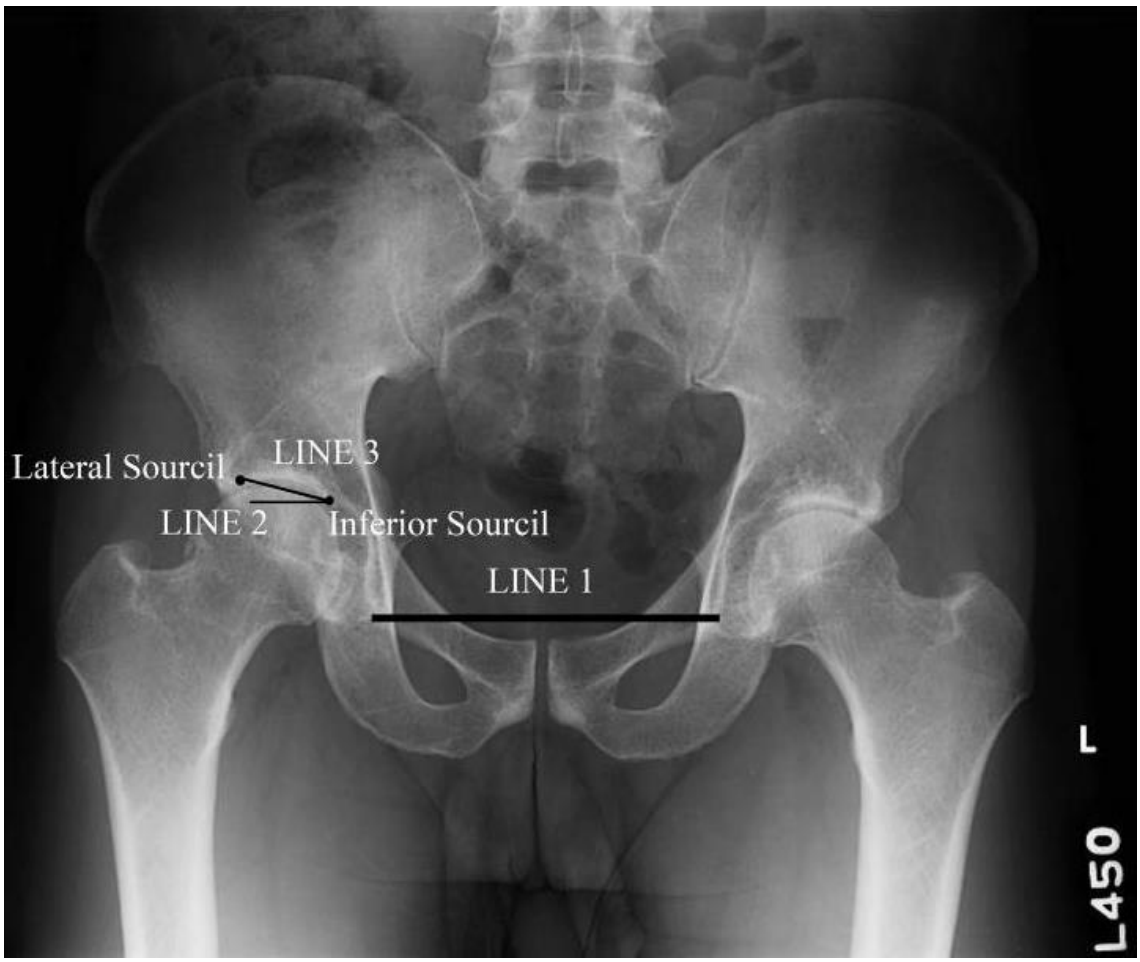

Figure 2-8: Representative technique for calculation of the Tönnis angle (JC Clohisy, A Systematic Approach to the Plain Radiographic Evaluation of the Young Adult Hip. 2008)

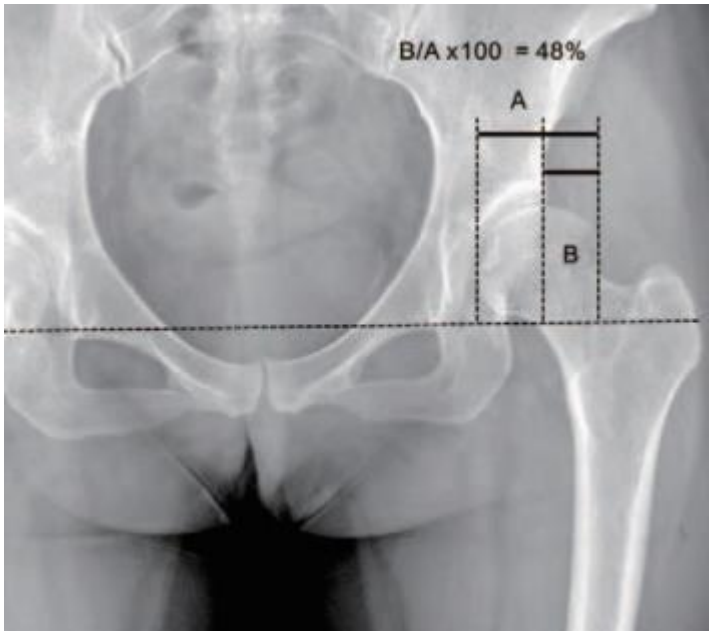

Figure 2-9: Representative technique for calculating the femoral head extrusion index. (SJ Lim, Plain Radiography of the Hip: A Review of the Radiographic Techniques and Image Features. 2015)

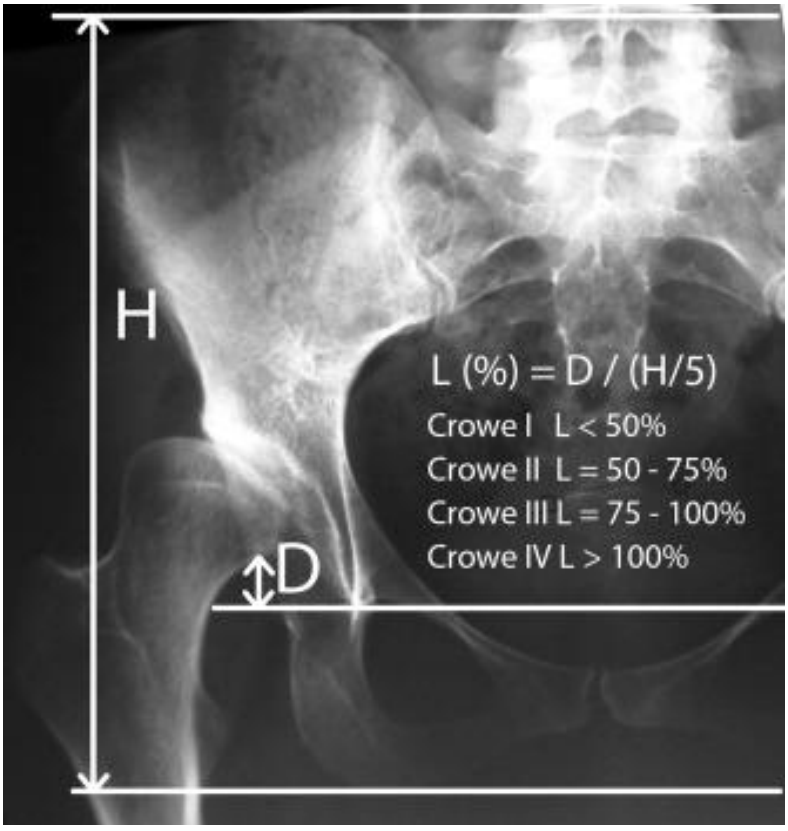

Figure 2-10: Crowe classification measurement with H representing height of the pelvis and D representing the distance from the inferior acetabular wall to the inferior portion of the femoral head neck junction (A Clavé, Influence of experience on intra- and inter-observer reproducibility of the Crowe, Hartofilakidis and modified Cochin classifications, 2016)

Grade	Radiographic features
0	- No signs of osteoarthritis
1	- Slight narrowing of joint space - Slight lipping at joint margin - Slight sclerosis of the femoral head or acetabulum
2	- Small cysts in the femoral head or acetabulum - Increasing narrowing of joint space - Moderate loss of sphericity of the femoral head
3	- Large cysts - Severe narrowing or obliteration of joint space - Severe deformity of the femoral head - Avascular necrosis

Table 2-1: Tönnis grading scale of hip osteoarthritis (B Kovalenko, Classifications in Brief: Tönnis Classification of Hip Osteoarthritis, 2018)

Figure 2-11: Pavlik Harness (A Besselaar, AO Surgery Reference, 2020)

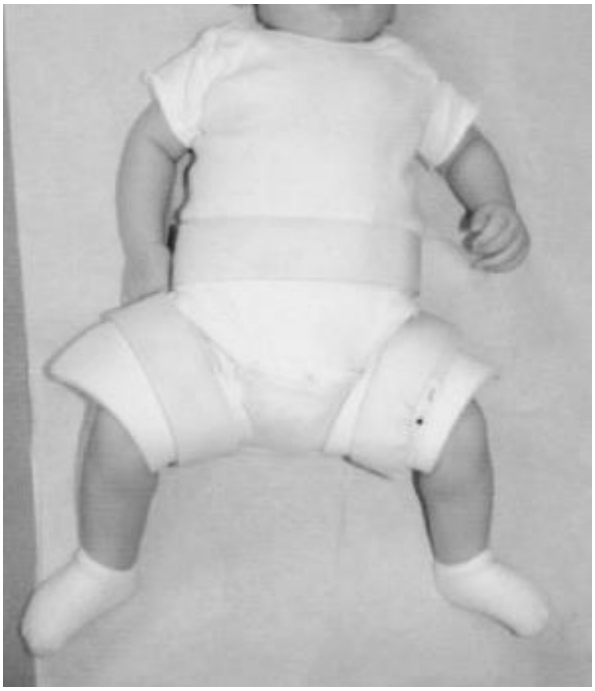

Figure 2-12: Fixed abduction brace (D Hedequist, Use of an Abduction Brace for Developmental Dysplasia of the Hip After Failure of Pavlik Harness Use, 2003)

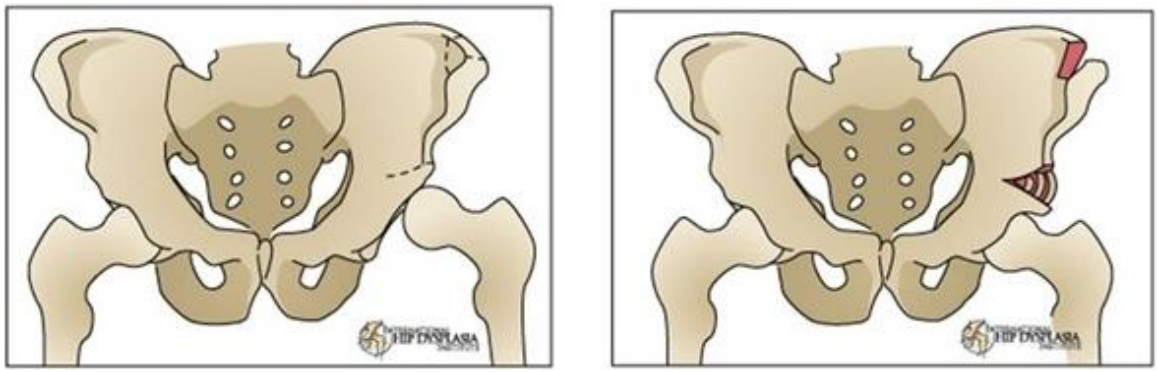

Figure 2-13: Representation of the Pemberton osteotomy. (International Hip Dysplasia Institute)

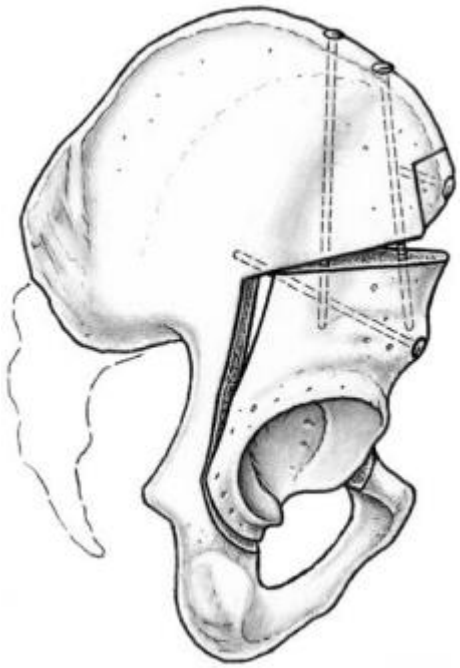

Figure 2-14: Representation of the periacetabular osteotomy (SD Steppacher, Mean 20-year Followup of Bernese Periacetabular Osteotomy, 2008)

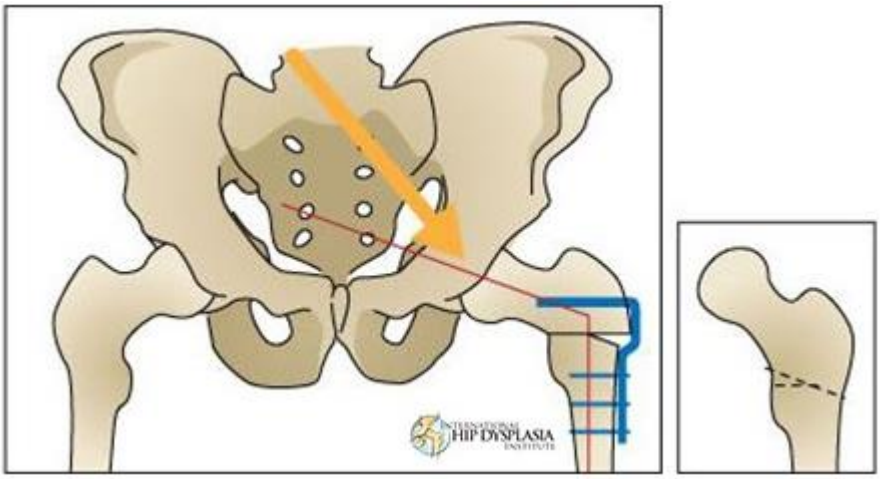

Figure 2-15: Representation of the Varus De-Rotational Osteotomy (VDRO) (International Hip Dysplasia Institute)

CHAPTER 3:

EFFECTS OF THE ACETABULAR LABRUM AND LIGAMENTUM TERES INTEGRITY ON *EX VIVO* HIP KINEMATICS

Research Objective and Hypothesis:

Best current evidence suggests that key soft tissues which stabilize the hip joint, such as the ligamentum teres and acetabular labrum, are mechanically overloaded when developmental dysplasia of the hip (DDH) progresses to symptomatic disease.[1–3] Recent biomechanical studies focused on DDH have employed simulation-based programs targeting the acetabular labrum as a primary stabilizing and load bearing structure in the hip.[4,5] However, the ligamentum teres of the femoral head has become more recognized as another important stabilizer of the hip with respect to DDH. The increased attention on the ligamentum teres has largely been driven by the growing use of arthroscopic surgery to assess and treat disorders of the hip.[6–8]

While simulation-based studies have provided important information on DDH-related biomechanics, they are limited by assumptions that do not address the variation in patient anatomy and pathology.[9,10] Using finite element analysis, the labrum has been reported to experience 2.8 to 4.0 times more load to maintain the position of the femoral head within the acetabulum in hips with DDH compared to hips without DDH.[1] Using a simulation of a healthy hip in the standing position, the insertion point of the ligamentum teres was determined to experience a high degree of stress, suggesting that the ligamentum teres may provide stability to the

joint when the hip is loaded.[11] Therefore, investigating the effects of the labrum and ligamentum teres on hip stability using cadaveric specimens under clinically relevant hip movements is needed to validate or refute the current simulation data and further characterize the roles of these structures in hip health and disease, including DDH.

Hip stability is typically measured using the translation of the femoral head relative to the neutral position of the hip. Previous MRI studies have reported that total femoral head translations for healthy hips are only approximately 1 mm when the hip was placed in 50° flexion, 40° abduction, and 60° external rotation from the neutral position.[12] When a hip joint translates outside of this range, the ligamentum teres and labrum are relatively overloaded[12]. However, the contributions from each tissue to hip stability during functional movements of daily living have not been fully elucidated. Therefore, the objective of this experiment was to further characterize the roles of these two key stabilizing tissues in the hip during flexion and range of motion movements of abduction/adduction and internal/external rotation. We hypothesized that resection of acetabular labrum, ligamentum teres, and both tissues would be associated with significant differences in femoral head translations while considering the specimen and side of the hip as random affects. Further, we hypothesized that resection of acetabular labrum, ligamentum teres, and both tissues would be associated with significant changes in joint torques through flexion and range of motion movements.

Materials and Methods:

Specimen Procurement and Preparation:

In accordance with institutional review board policies and guidelines for use of cadaveric specimens, full pelvis specimens (Science Care, Inc., Phoenix, AZ) were sectioned from the 3rd lumbar vertebra to mid-femur with all structures intact. Full pelvises (n=5, 4 male, 1 female, mean age 38.8 years, range 19-51 years) were stored at -20°C. One randomly selected hemipelvis was utilized for protocol optimization and not used for subsequent testing, leaving 5 left and 4 right hips for data collection. Specimens were thawed and all surrounding soft tissue was removed leaving the joint and joint capsule intact. Utilizing the NDI Optotrak system (Waterloo, Ontario, CA) the bilateral anatomical landmarks **(Figure 3-1)**, anterior superior iliac spine, posterior superior iliac spine, medial femur, lateral femur, and self-selected fiducial points on the pelvis and femur were digitized. These values were then loaded into the SimVitro software (Version 4.2.0.64; Cleveland Clinic) to create a hip joint coordinate frame.

Full pelvises were split into their respective hemipelvis at the sacroiliac joint and the pubic symphysis. The iliac crest clamped into a custom-made 3D printed clamp. The clamped iliac crest and distal portion of the femur were potted in 2.5-inch diameter aluminum pots to a depth of approximately two inches using a 70°C low melting point Woods metal alloy. Two drill bits were placed through the pot and into the femur to provide maximum stability for the hemipelvis within the pots **(Figure 3-2).** Potted specimens were placed in high precision memory-lock clamps allowing the specimen to be secured in only one orientation. The memory lock clamps were connected to two Omega 160 IP65 force/torque sensor load cells

(ATI Industrial Automation, Apex, NC) and attached to the KUKA (Augsburg, DE) Kr300 R2500 Ultra robotic testing system.

Optotrak markers, integrated into the Optotrak Certus system, were attached to the previously selected fiducial positions. With the femur secured to the robot, the pelvis was unattached and manually moved through the passive flexion/extension, abduction/adduction, and internal/external rotation movements during a 30-second motion capture. For each 30-second motion capture, 10 points are collected per second from the fiducial markers to precisely calculate the center of rotation for the specimen. Once the center of rotation root mean square error (RMSE) of <0.25 mm was obtained, well below the maximal expected error of up 1 mm[13], the clamp was reattached to the robot and the specimen was placed into a neutral position, based on the hip and femur orientation, by a trained physical therapist. Each specimen was placed under 10 N of superior and 10 N of medial controlled forces, to maintain femoral head and acetabulum contact through movements, as previously described.[14] While moving to 90° flexion from neutral, without any internal/external rotations or abduction/adduction, the trained physical therapist observed the movement to confirm the cadaver pelvis did not have any abduction/adduction or internal/external rotation movements that were not commanded.

Testing Protocol:

To test our hypothesis regarding hip stabilizing tissues, four hip statuses were considered (1) the native state with all intra-articular structures intact, (2) the

complete removal of the ligamentum teres using a medial approach, (3) complete removal of the labrum using a lateral approach, and (4) a combination of the removal of both the ligamentum teres and labrum using the described approaches. For the fourth hip status, the order of tissue removal was randomly chosen with the ligamentum teres being removed first four times and the labrum removed first five times. The removal of each structure was followed by a capsular repair with 2-0 fiberwire by a human orthopaedic technologist-surgery certified individual.

Each specimen experienced a full range of motion test. This included various internal/external rotation and abduction/adduction specific to the flexion angles of 0°, 30°, 60°, and 90° along with extension of 10° as outlined previously.[14] Clinically applicable tests were conducted, as described in Chapter 2, and were intended to replicate the range of motion testing completed by physicians as part of the process to detect hip pathologies. These include FADIR (flexion 90°, adduction 25°, and internal rotation 15°) and FABER (flexion 90°, abduction 55°, and external rotation 25°) motions.[15,16] **(Table 3-1)** The order of tested movements for each specimen was randomized using the randomization function within SimVitro software. Each movement tested completed 10 cycles, where each cycle is defined as a full movement through one range of motion. Data points for femoral head translations and joint torques were collected for analysis at cycle 1, 2, 5, and 10 to account for potential tissue stiffness and relaxation across multiple cycles. For all motions, a termination criterion was applied to avoid destructive testing of the joint. This was defined as a torque threshold of 5 Nm, which represents end of range of motion as described previously.[17,18]

Statistical Analysis:

Translations and torques were collected from SimVitro software (Version 4.2.0.64; Cleveland Clinic) in the “State Joint Coordinate System” tab of the data and only non-missing data were considered for analysis. A combination of all three planes of displacement were analyzed. Initially, a multivariate ANOVA (MANOVA) was conducted, using the three planes of displacement as the outcome of interest with specimen and specimen side as random effects. The MANOVA was used to determine fixed effects for the removal of the intra-articular tissues compared to native, the flexion angles, and range of motion movements of abduction, adduction, internal, and external rotation. Interactions between the flexion angles and range of motion movements, with respect to the changes in intra-articular tissues present, were also considered. Outliers that were more than three times the IQR at the 25^{th} and 75^{th} quartile were excluded. Normality assumptions were checked using Q-Q plots and the Shapiro-Wilks test. Estimated marginal means with Bonferroni correction were used to check post-hoc, pairwise differences between significant fixed factors. Effect size for each factor was calculated as the ratio of explained variance such that an effect size of 0.06 is considered as medium effect, while an effect size of 0.14 is considered as large effect.[19] Transformations for displacement measurements were considered for analysis but ultimately not utilized due to problematic interpretation for clinical understanding. Alternatively, total displacement of the femoral head was transformed into a polar coordinates system and graphed for visual comparisons.

A multivariable linear mixed model (LMM) was utilized, separately, for outcomes extension, abduction, and external rotation torque measurements. Random effects were the cycle by specimen while fixed effects included the specimen and specimen side. Fixed effects tested were the removal of the intra-articular tissues compared to native, the flexion angles, and range of motion movements of abduction, adduction, internal, and external rotation with all two-way interactions. LMM models utilized a stratified 10-fold cross-validation. The performance is determined by using an adjusted R-squared with a penalization for including additional independent variables. The model performances were evaluated by comparing the R-squared and adjusted R-squared such that a small difference between the R-squared (obtained from the training data) and predicted adjusted R-squared (obtained from the testing data) indicates no overfitting. Homoscedasticity and normality assumptions were checked through residual plots and Q-Q plots. Any significantly different interactions for hip statuses and movements from the LMM had the change in translations calculated with respect to the neutral position with no flexion. R version 4.1.3 with RStudio and packages plotly, emmeans, and rstatix were used for all analyses.

Results:

MANOVA:

Biomechanical testing resulted in 2916 separate, but not independent, observations. One-hundred and six observations with missing values were excluded from the MANOVA model, and 218 extreme outliers were removed.

MANOVA assumptions for normality were not met, even after exclusion of outliers. Only significant factors with large effect sizes were considered statistically significant. For anterior translation, the flexion angle was significantly different ($p<0.0001$) with a large effect size (0.19). For superior translation, there were no significant differences with large effect sizes. For lateral translation, there was a significant difference in hip status ($p<0.0001$) with a large effect size (0.14), **(Table 3-2)**

A post hoc analysis was conducted for hip status in the lateral translation. The ligamentum teres removal was significantly different from the native status ($p<0.0001$) and the removal of the labrum ($p<0.0001$). The removal of both the ligamentum teres and the labrum was significantly different from the native status ($p<0.0001$), the removal of the labrum ($p=0.0007$), and the removal of the ligamentum teres ($p=0.0009$). In addition, a post hoc analysis was completed on the hip flexion angle for anterior translation. In the pairwise comparison of flexion angle with anterior translation all comparisons between 0°, 30°, 60°, and 90° flexion and 10° extension were significantly different from one another. **(Table 3-3)**

Polar Coordinate Systems:

The polar coordinate system was used to visualize total positive displacement of femoral head translations. These figures indicated visual increases in total positive displacement of femoral head translations as flexion angles increased (**Figure 3-3A**, yellow (0°), blue (10° extension), orange (30°

flexion), and purple (60° flexion)). There were no visual differences in the displacement when considering hip status, as indicated by the different symbols (**Figure 3-3A**, native (circle), labrum remove (square), ligamentum teres removed (diamond), or the removal of the labrum and ligamentum teres (cross)). There were also no visual differences in the flexion angle or the hip status in the theta plane, i.e. the angle between lateral translation and overall displacement. Similarly, no visual differences were observed in the phi plane, i.e. the angle between anterior translation and overall displacement.

With the difference in range of motion movements (**Figure 3-3B**, green (neutral), yellow (abduction), blue (adduction), blue (internal rotation), and orange (external rotation)), there was no visual difference for each movement in the angular displacement (theta) or for each status. However, a slight visual increase in the theta was visually observed for the ligamentum teres ("lt", green symbols), indicating angular displacement but there was no visual difference in the range of motion movements.

Linear Mixed Model: **(Table 3-5)**

One-hundred and eighty-two observations with missing values were excluded from the linear mixed models. The predicted adjusted R_2 and adjusted R_2 were similar indicating the LMM were not overfit, and the linearity, homoscedasticity, and normality were checked; there was no violation of assumptions. **(Table 3-4)**

At 10° of extension the removal of the ligamentum teres had a significant decrease in the extension torque estimate of 0.68 Nm (std. error: 0.19) while the removal of the labrum had a significant decrease in the abduction torque estimate of 1.18 Nm (std. error: 0.22) and external torque estimate of 0.64 Nm (std. error: 0.19). At 10° of extension the removal of ligamentum teres and labrum had a significant decrease in external torque estimate of 0.39 Nm (std. error: 0.16). At 60° of flexion the removal of the ligamentum teres had a significant decrease in extension torque estimate of 0.46 Nm (std. error: 0.16) while the removal of the ligamentum teres and labrum had a significant decrease in extension torque estimate of 0.26 Nm (std. error: 0.12). At 90° of flexion the removal of the ligamentum teres had a significant decrease in extension torque estimate of 0.57 (std. error: 0.14) while the removal of the ligamentum teres and labrum had a significant decrease in extension torque of 0.39 Nm (std. error: 0.10) and increase of abduction torque of 0.34 Nm (std. error: 0.15).

With adduction movements the removal of the labrum had a significant increase in abduction torque estimate of 0.49 Nm (std. error: 0.21) while the removal of the ligamentum teres and the labrum had a significant increase in abduction torque estimate of 0.38 Nm (std. error: 0.18). With external rotation movements the removal of the ligamentum teres had a significant increase in extension torque estimate of 0.43 Nm (std. error: 0.15) while the removal of the labrum had a significant decrease in external rotation torque of 0.33 Nm (std. error: 0.16).

With extension the removal of the ligamentum teres resulted in femoral head translations of 0.04 mm anteriorly, 0.79 mm superiorly, and 0.23 mm laterally while the removal of the labrum result in femoral head translations of 0.01 mm anteriorly, 0.69 mm inferiorly, and 0.39 mm laterally. With extension and the removal of the ligamentum teres and labrum resulted in femoral head translations of 0.2 mm anteriorly, 0.09 mm superiorly, and 0.38 mm laterally. With 60° of flexion the removal of the ligamentum teres resulted in femoral head translations of 2.92 mm anteriorly, 0.2 mm inferiorly, and 0.54 mm laterally while the removal of the ligamentum teres and labrum resulted in femoral head translations of 0.87 mm anteriorly, 0.07 mm inferiorly, and 0.92 mm laterally. With 90° of flexion the removal of the ligamentum teres resulted in femoral head translations of 4.55 mm anteriorly, 1.25 mm superiorly, and 0.25 mm laterally while the removal of the ligamentum teres and labrum resulted in femoral head translations of 1.49 mm anteriorly, 0.07 mm superiorly, and 0.33 mm laterally.

With adduction movements the removal of the labrum resulted in femoral head translations of 0.09 mm anteriorly, 0.72 inferiorly, and 1.17 mm laterally while the removal of the ligamentum teres and labrum resulted in femoral head translations of 0.14 mm anteriorly, 0.28 mm inferiorly, and 1.04 mm laterally. With external movements the removal of the ligamentum teres resulted in femoral head translations of 0.36 mm anteriorly, 0.49 mm inferiorly, and 0.23 mm medially while the removal of the labrum resulted in femoral head translation of 0.71 mm posteriorly, 1.23 mm inferiorly, and 0.59 mm laterally.

Discussion:

The first hypothesis was rejected; while the state of the hip affected femoral head translations, the combined effects of the state of the hip with flexion and range of motion movements did not have statistical significance in any plane of translation. With this MANOVA analysis, there were also assumptions that were violated, so caution must be taken when interpreting the results as there was a large degree of variability within the data. The second hypothesis was accepted as resections of acetabular labrum, ligamentum teres, and both tissues were associated with significant changes in joint torques associated with the state of the hip in combination with either flexion angle or range of motion movements.

In the MANOVA analysis, the large degree of variability in the data may be partially described by the variations in joint morphology among specimens.[20–24] This suggestion is supported by the variability in polar coordinate data as there were significant differences for anterior translation among flexion angles and for total positive displacement with increase flexion. These differences may be attributed to the shape of the femoral head, described as a conchoid shape and not a perfect sphere.[25,26]

There were significant differences for lateral translation of the femoral head based on the state of the hip. The resection of both the ligamentum teres and labrum was associated with significantly different lateral translation when compared to the native intact hip and to resection of the labrum or ligamentum teres alone. The resection of the ligamentum teres was associated with significant differences in lateral translation when compared to resection of the labrum and to

the native intact hip, while resection of the labrum was not associated with significant differences in lateral translation from the native intact hip. These cadaveric biomechanical testing data suggest that the ligamentum teres may play a more prominent role in hip joint stability during movements of daily living, as previously reported.[27,28] However, the MANOVA results indicated that the laterality (left versus right) of the hip tested, as well as each cadaveric specimen, were significantly different for lateral translation such that conclusions must be tempered based on these violations of the statistical analysis.

The multivariable linear mixed model results indicated that the ligamentum teres predominantly provided anterior stability with increased hip flexion (60° and 90°). These results were similar to those reported in previous descriptive anatomical studies and computer modeling studies.[28,29] In the present study, ligamentum teres was also noted to contribute to inferior stability through adduction and external rotation movements, which has also been described in a previous study.[28] Additionally, the multivariable linear mixed model results indicated that the labrum primarily provides lateral stability when the hip is placed in adduction. Typically, patients with labral damage have pain in adduction, limiting their range of motion, which corresponds well with the biomechanical testing data from this experiment as labral deficiency may allow for abnormal translations that cause pain and limit motion.[30] The labrum was also noted to provide inferior stability when the hip was placed in external rotation consistent with a previous study that reported that external rotation of the hip places increased strain on the posterior compartment of the joint.[31]

Taken together, the results of this experiment indicate that the ligamentum teres plays important roles in hip stability, especially at higher angles during flexion movements. The ligamentum teres appeared to provide predominantly anterior stability based on anterior translations far exceeding the 1 mm of displacement reported for healthy hips.[12] These cadaveric biomechanical testing data also indicate that the acetabular labrum plays important roles in hip stability. When labrum-deficient hips were tested in adduction, femoral head translation slightly exceeded the 1 mm of displacement reported for healthy hips.[12]

While the data from this study indicated that both the labrum and the ligamentum teres serve important roles to stabilize the hip, there are limitations to acknowledge. As discussed above, the assumption of normality for the MANOVA analysis was violated. Importantly, the joint capsule was disrupted and repeatedly repaired between biomechanical testing sessions for each hip status, which this may have confounded results based the intact capsule's roles in hip stability.[32] Further, the surrounding musculature of the hip, which also contributes to dynamic hip stability, was removed in an attempt to clearly delineate the roles of the two targeted intra-articular soft tissue structures.[33,34] Based on specimen variation, which mimics the clinical scenario, it will be important in future studies to include analysis of the individual joint morphologies to better account for relevant differences based on patient variables included size, sex, and laterality.[20–24] In addition, differences in the multivariable linear mixed model based on order of tissue resection should be addressed by randomization resection and testing order in future studies.

In conclusion, the data from this study indicate that the acetabular labrum and the ligamentum teres each play unique roles for hip joint stability during activities of daily living. The acetabular labrum appears to provide lateral stability during adduction movements and inferior stability during external rotation movements. The ligamentum teres appears to provide anterior stability when the hip is flexed past 60°. Taken together, these findings suggest that each structure is important for hip stability and joint health with the most significant concern for pathological joint instability occurring when both soft tissues are deficient. This investigation regarding the effects of the labrum and ligamentum teres on hip stability using cadaveric biomechanical testing of hip movements contributes clinically relevant data on the roles of these structures in hip health and disease, including developmental dysplasia of the hip.

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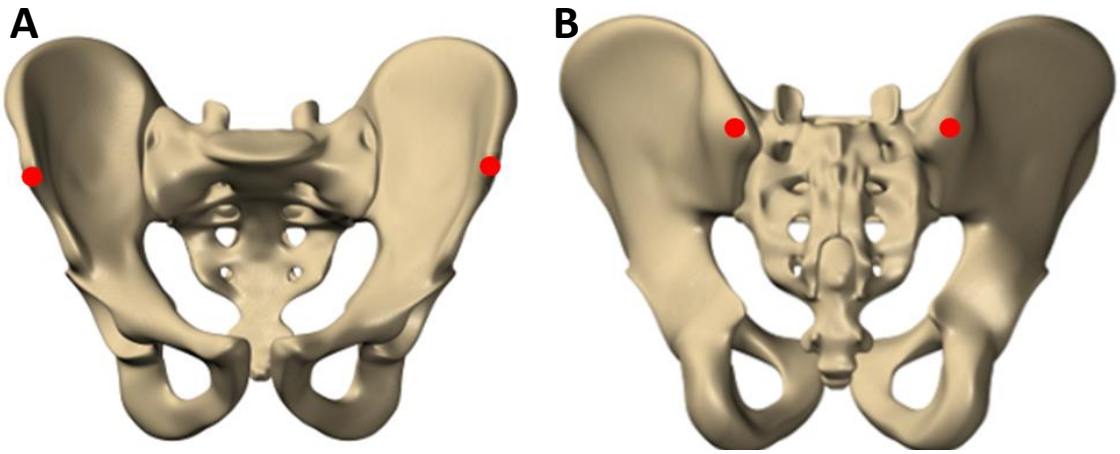

Figure 3-1: Anatomical landmarks on the pelvis identified for specimen digitization A.) Anterior view of the pelvis with the anterior superior iliac spine indicated by the red dots, B.) Posterior view of the pelvis with the posterior superior iliac spine indicated by the red dots.

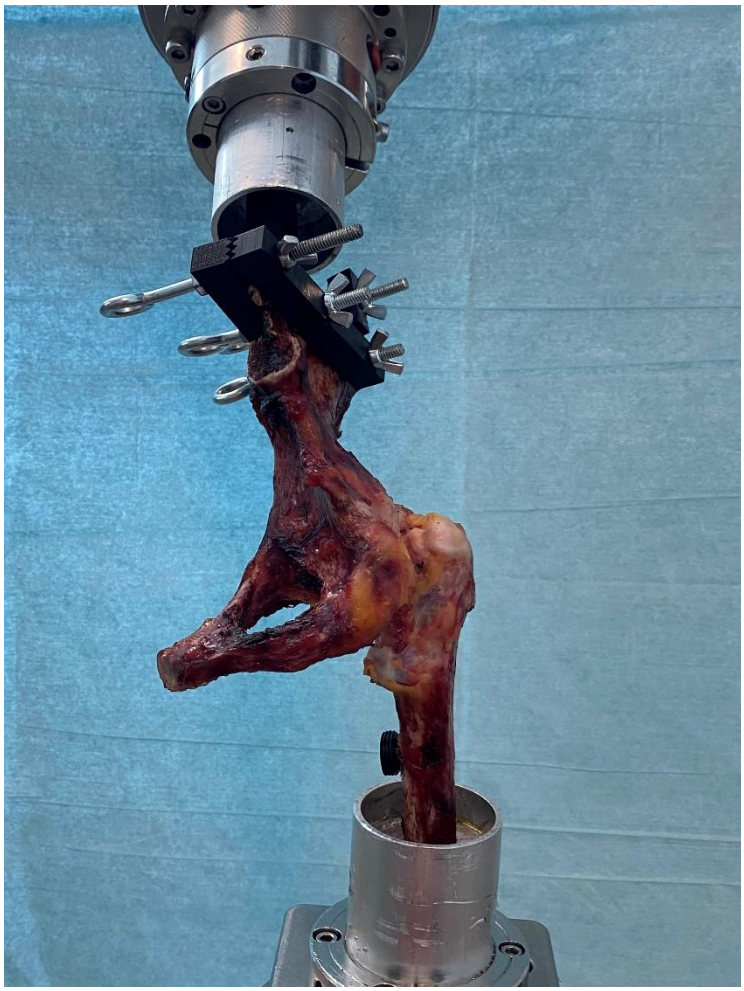

Figure 3-2: Clamped and potted hemipelvis

Movement	Degrees	Clinical Movement	Internal Rotation	External Rotation	Abduction	Adduction
Extension	10°		20°	20°	25°	6°
Neutral	0°		30°	30°	40°	10°
	30°		40°	40°	40°	25°
	60°		25°	50°	45°	30°
Flexion	90°		20°	45°	45°	20°
	90°	FABER		25°	55°	
	90°	FADIR	15°			25°

Table 3-1: Range of motion movements applied to the specimens at each tested flexion angle and the angle of movements for the clinically

Anterior Translation						
Factors	**Df**	**Sum Sq**	**Mean Sq**	**Effect size**	**F value**	**Pr(>F)**
State	3	1095.11	365.04	0.03	51.14	0
Flexion *	4	6610.28	1652.57	0.19	231.5	0
ROM	6	295.76	49.29	0.01	6.91	0
Sample	4	3084.2	771.05	0.09	108.01	0
Side	1	3739.6	3739.6	0.11	523.87	0
State & Flexion	12	570.69	47.56	0.02	6.66	0
State & ROM	18	520.13	28.9	0.02	4.05	0
Flexion & ROM	16	312.85	19.55	0.01	2.74	0.0002
State, Flexion & ROM	48	480.64	10.01	0.01	1.4	0.0359
Superior Translation						
State	3	410.33	136.78	0.02	20.67	0
Flexion	4	1601.39	400.35	0.08	60.51	0
ROM	6	560.45	93.41	0.03	14.12	0
Sample	4	138.54	34.63	0.01	5.23	0.0003
Side	1	206.07	206.07	0.01	31.15	0
State & Flexion	12	118.28	9.86	0.01	1.49	0.1204
State & ROM	18	132.91	7.38	0.01	1.12	0.3288
Flexion & ROM	16	365.5	22.84	0.02	3.45	0
State, Flexion & ROM	48	265.3	5.53	0.01	0.84	0.7827
Lateral Translation						
State *	3	1634.15	544.72	0.14	250.66	0
Flexion	4	117.82	29.46	0.01	13.55	0
ROM	6	475.39	79.23	0.04	36.46	0
Sample *	4	1685.34	421.33	0.15	193.89	0
Side *	1	1579.51	1579.51	0.14	726.85	0
State & Flexion	12	146.77	12.23	0.01	5.63	0
State & ROM	18	53.85	2.99	0	1.38	0.1323
Flexion & ROM	16	38.86	2.43	0	1.12	0.3317
State, Flexion & ROM	48	128.68	2.68	0.01	1.23	0.1316

Table 3-2: MANOVA results for the planes of translation associated with the state, flexion, range of motion, sample, hip side, and the combinations of state, flexion, and range of motion. *=significant ($p<0.05$) with a large effect size (>0.14)

Post-Hoc Results				
State	**Native**	**Labrum**	**Ligametum Teres**	
-Labrum	1.00			
-Ligamentum Teres	<0.001 *	<0.001 *		
-Ligamentum Teres & Labrum	<0.001 *	0.001 *	0.001 *	
Flexion (°)	**0**	**-10**	**30**	**60**
-10	<0.001 *			
30	<0.001 *	<0.001 *		
60	<0.001 *	<0.001 *	<0.001 *	
90	<0.001 *	<0.001 *	<0.001 *	<0.001 *

Table 3-3: MANOVA results with post-hoc analysis and Bonferroni correction results for the state of the hip with lateral translation and the flexion angles of the hip with anterior translation *=significant ($p<0.05$)

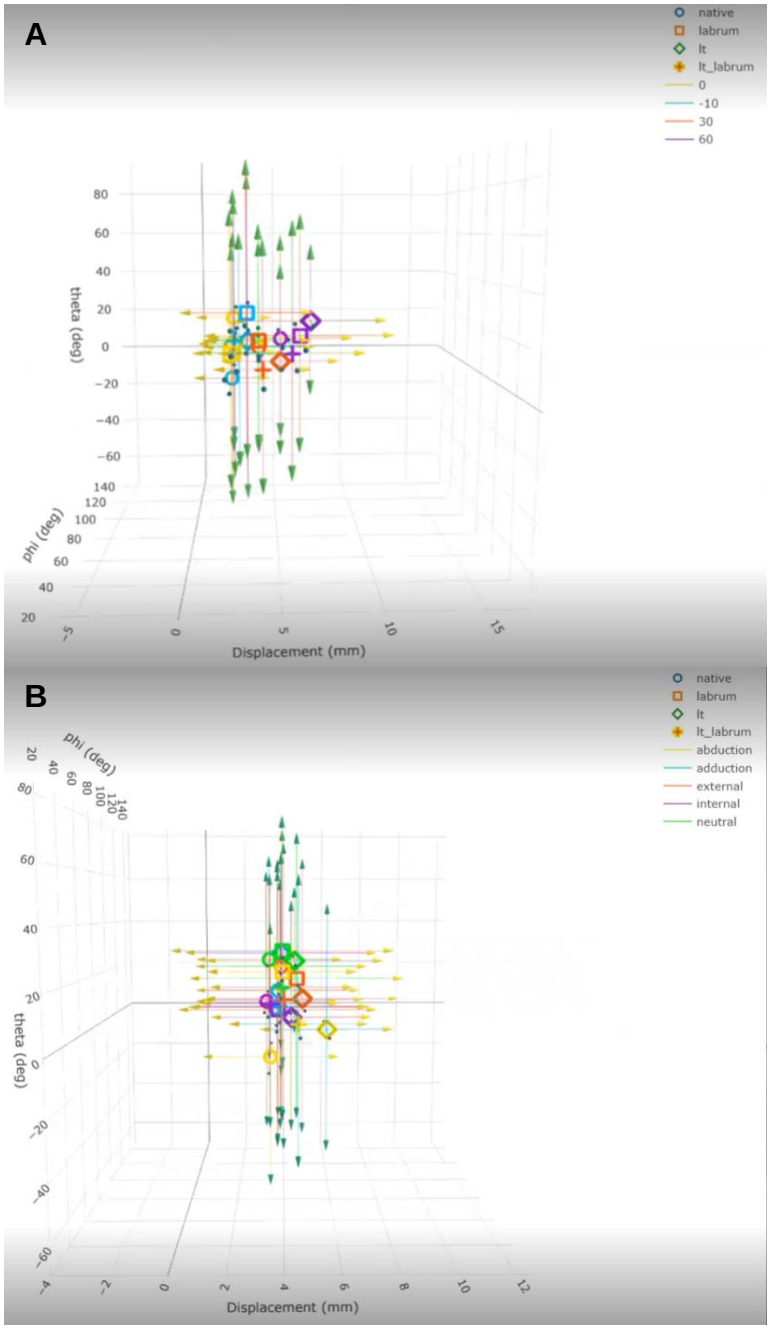

Figure 3-3: Mean and standard deviations for total displacement using a spherical coordinate system. Mean values are denoted by colored symbols, lines with arrows indicate the amount and direction for standard deviations, across A) flexion angles and hip status and B) range of motion movements and hip status.

	R_2	Adjusted R_2	Predicted R_2	Adjusted Predicted R_2
Extension	0.5664	0.5619	0.5626	0.5581
Abduction	0.5891	0.5835	0.5615	0.5555
External Rotation	0.5435	0.5394	0.5259	0.5217

Table 3-4: Model performances evaluation for overfitting using R-squared (obtained from the training data) and predicted adjusted R-squared (obtained from the testing data)

		Torque Estimates (Nm)			Translations (mm)		
Movement	Status	Extension	Abduction	External	Anterior	Superior	Lateral
Extension 10°							
	-Ligamentum teres	-0.68 (std. error: 0.19)			0.04	0.79	0.23
	-Labrum		-1.18 (std. error: 0.22)	-0.64 (std. error: 0.19)	0.01	-0.69	0.39
	-Ligamentum teres & Labrum			-0.39 (std. error: 0.16)	0.2	0.09	0.38
Flexion 60°							
	-Ligamentum teres	-0.46 (std. error: 0.16)			2.92	-0.2	0.54
	-Ligamentum teres & Labrum	-0.26 (std. error: 0.12)			0.87	-0.07	0.92
Flexion 90°							
	-Ligamentum teres	-0.57 (std. error: 0.14)			4.55	1.25	0.25
	-Ligamentum teres & Labrum	-0.39 (std. error: 0.10)	0.34 (std. error: 0.15)		1.49	0.07	0.33
Adduction							
	-Labrum		0.49 (std. error: 0.21)		0.09	-0.72	1.17
	-Ligamentum teres & Labrum		0.38 (std. error: 0.18)		0.14	-0.28	1.04
External							
	-Ligamentum teres	0.43 (std. error: 0.15)			0.36	-0.49	-0.23
	-Labrum			-0.33 (std. error: 0.16)	-0.71	-1.23	0.59

Table 3-5: Linear mixed model results for significant changes in estimate torques and their respective mean differences in translations compared to the native state at 0° flexion.

CHAPTER 4:

METABOLIC ANALYSIS OF OSTEOARTHRITIC TISSUES IN THE HIP

Research Objective and Hypothesis:

Current knowledge on the metabolic activity of tissues in the hip - acetabular labrum, ligamentum teres, articular cartilage of the femoral head, and synovium - is limited. Previous studies assessing the metabolic profile of intra-articular tissues of the knee have indicated that each tissue has unique metabolic responses that contribute to joint homeostasis and the development and progression of osteoarthritis (OA).[1–4] While the hip and knee have similar intra-articular tissue structures and are commonly affected by OA, previous studies have indicated differences between the knee and the hip for articular cartilage epigenetics and the composition of the synovial fluid of osteoarthritic patients.[5,6] Therefore, data from studies assessing changes in tissue metabolism associated with OA development in the knee may not be reflective of changes that occur to similar tissues in the hip. Therefore, it is important to assess the tissue-specific metabolic responses of the acetabular labrum, ligamentum teres, synovium, and femoral head cartilage recovered from osteoarthritic hips to determine how each structure responds during OA development and progression. Understanding how each tissue contributes to the development and progression of hip OA may provide tissue-specific targets to slow the progression to OA. It was hypothesized that the acetabular labrum, ligamentum teres, synovium, and femoral head cartilage, recovered from patients undergoing total hip arthroplasty (THA) for OA, would

have unique metabolic profiles based on significant differences in the production of biomarkers related to degradative enzyme production and regulation, inflammatory signaling, and growth factors by the tissues during ex vivo culture.

Materials and Methods:

Tissue Collection and Culture:

With IRB approval (#2016684) and informed patient consent, intra-articular tissues that would normally be discarded were recovered from patients undergoing total hip arthroplasty (n=25, 14 female, 11 male, mean age of 58.5 years (range 33.6 to 79.9 years), mean BMI of 33.2 (range 21 to 44.7 BMI)). The acetabular labrum (n=13), ligamentum teres (n=13), synovium (n=23), and femoral head cartilage (n=24) were collected **(Table 4-1)**, sectioned into approximately 10 mm full thickness pieces, and placed in 5 mL of Dulbecco's Modified Essential Medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA). DMEM was supplemented with 1mM sodium pyruvate, 2mM L-glutamine, 0.5mg/mL ascorbic acid, 1 x MEM N-E Amino Acid solution, 1% insulin transferrin selenium (ITS premix: BD Biosciences, Bedford, MA, USA), and 1x penicillin-streptomycin-amphotericin B (all components from Invitrogen Co., Carlsbad, CA, USA unless otherwise specified). Tissues were cultured in a 6-well plate for three days at 37°C with 5% CO2 and 95% humidity. On day three of culture, the media was collected and stored at -20ºC until used for biomarker testing described below. After culture, the wet weight of the tissue was determined.

Protein Analysis:

Multiplex Luminex assays were used to test day 3 culture media for matrix metalloprotease (MMP)-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9 , MMP-13 using the Performance Human MMP Magnetic Panel (R&D Systems, Minneapolis, MN, USA); tissue inhibitor of matrix metalloproteases (TIMP)-1, TIMP-2, TIMP-4 using the Human TIMP panel 2 (Millipore, Billerica, MA, USA); and interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10, growth related oncogene (GRO)-α, monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage inflammatory proteins (MIP)-1α, MIP-1β, platelet derived growth factor (PDGF)-AA, regulated on activation normal T expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF) using the Human Cytokine Magnetic Panel (Millipore, Billerica, MA, USA). The media were tested for a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 4 using the Human ADAMTS4 DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) and Prostaglandin E_2 (PGE_2) using the Prostaglandin E_2 Express ELISA (Cayman Chemical Co., Ann Arbor, MI). Media were also analyzed for nitric oxide using the 2,3-Diaminomaphthalene (DAN) assay[7], glycosaminoglycans (GAG) using the dimethylmethylene blue (DMMB) colorimetric assay[8], and MMP activity using the 520 MMP FRET Substrate XIV[9] (Anaspec, Fremont, CA). For statistical analysis, biomarker concentrations were standardized to the tissue wet weight in grams to account for variability in tissue explant size.

Statistical Analysis:

Statistical analysis was completed in SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp). To determine metabolic differences between tissue types, a Kruskal-Wallis test was used for statistical analysis, and if warranted, followed by a Dunn test for pairwise comparison with Bonferroni correction for multiple comparisons. Significance was set with a two-sided test at $p<0.05$. Figures and tables were created in Microsoft Excel (Microsoft Corp. Version 16.0. Redmond, WA).

Results:

Degradative Enzyme-Related Biomarkers:

The median production of MMP-3 **(Figure 4-1C)** by the cartilage, 9057 ng/mL/g (range: 7612-13901 ng/mL/g), was significantly higher than the synovium ($p=0.012$), 2998 ng/mL/g (range: 2435-4430 ng/mL/g) and the ligamentum teres ($p=0.021$), 3444 ng/mL/g (range: 2419-4430 ng/mL/g). The median production of MMP-13 **(Figure 4-1G)** by the cartilage, 350 ng/mL/g (range: 67.75-526.03 ng/mL/g), was significantly higher than the synovium ($p<0.001$), 4.73 ng/mL/g (range: 0.88-44.43 ng/mL/g), and the ligamentum teres ($p=0.012$), 6.29 ng/mL/g (range: 1.24-43.70 ng/mL/g). The median production of MMP-7 **(Figure 4-1D)** by the cartilage, 2829 pg/mL/g (range: 1825-5187 pg/mL/g), and the labrum, 2109 pg/mL/g (range: 1230-3993 pg/mL/g), were significantly higher compared to the synovium ($p<0.001$ and $p=0.008$), 500 pg/mL/g (range: 299.75-1748 pg/mL/g), and ligamentum teres ($p<0.001$ and $p=0.012$), 491 pg/mL/g (range: 338.07-1206

pg/mL/g). The median production of MMP-9 by the cartilage tissue, 0 pg/mL/g (range: 0-123.83 pg/mL/g), was significantly lower than the labrum (p=0.006) , 828 pg/mL/g (range: 507.68-1360 pg/mL/g), the synovium (p<0.001) , 1276 pg/mL/g (range: 994.41-1440 pg/mL/g), and the ligamentum teres (p<0.001) , 1003 pg/mL/g (range: 746.50-2012 pg/mL/g) **(Figure 4-1F)**. The median production of ADAMTS4 by the cartilage, 0 pg/mL/g (range: 0-29.19 pg/mL/g), was significantly lower than the synovium (p=0.008), 110.93 pg/mL/g (range: 68.72-146.23 pg/mL/g), and the ligamentum teres (p=0.003), 163.54 pg/mL/g (range: 155.09-191.90 pg/mL/g) **(Figure 4-1I)**.

Indicative of the differences in extracellular matrix composition, the median GAG released to the media **(Figure 4-1M)** from the cartilage, 22.25 mg/mL/g (range: 15.85-25.34 mg/mL/g), were significantly higher compared to the synovium (p<0.001), 4.95 mg/mL/g (range: 3.31-6.70 mg/mL/g), and the ligamentum teres (p=0.002), 7.72 mg/mL/g (range: 3.97-14.22 mg/mL/g). Because the DMMB assay cannot differentiate between GAG release due to synthesis or degradation, the mechanism responsible for increased GAG release to the media by cartilage samples is not known. While this data indicates that cartilage tissue produced significantly higher levels of some of the key MMPs assessed in this study, the level of median MMP activity in media from synovial samples, 1386 ng/mL/g (range: 641.69-3028 ng/mL/g), was significantly higher than the cartilage (p=0.003), 378.96 ng/mL/g (range: 183.10-639.12 ng/mL/g) **(Figure 4-1H)**. This indicates that, even though there was not a significant difference in the production of TIMPs between groups observed in this study, the cartilage tissue regulates

MMP activity better than synovial tissue during ex vivo culture. Because the cartilage tissue had lower MMP activity and lower ADAMTS4 production, it is possible the higher GAG release to the media is due to increased production by the cartilage tissue and not degradation due to degradative enzyme activity. **(Table 4-3)**

Inflammation-Related Biomarkers and Growth Factors:

The data from this study indicate differences in the inflammation related metabolic responses of cartilage and synovium. The synovium produced significantly higher levels of median IL-6 (p=0.016) , 11461 pg/mL/g (range: 7490-16295 pg/mL/g), **(Figure 4-2C)** compared to cartilage, 126 pg/mL/g (range: 23.83-8701 pg/mL/g), and median Gro-α (p=0.017), 4111 pg/mL/g (range: 3084-7888 pg/mL/g), **(Figure 4-2H)** compared to the cartilage, 246 pg/mL/g (range: 0-5043 pg/mL/g). Further, while the synovium produced significantly higher levels of median PGE2, 25050 pg/mL/g (range: 11501-60864 pg/mL/g), **(Figure 4-2F)** compared to the cartilage (p=0.004), 5160 pg/mL/g (range: 2042-7837 pg/mL/g), and the labrum (p=0.006), 4558 pg/mL/g (range: 1621-7248 pg/mL/g). The cartilage produced significantly higher levels of median NO, 0.67 pg/mL/g (range: 0.19-1.44 pg/mL/g), **(Figure 4-2G)** compared to the synovium (p=0.044), 0.25 pg/mL/g (range: 0.09-0.33 pg/mL/g), indicating a potentially important difference in how each tissue responds to inflammatory stimulus. Surprisingly, the cartilage tissue, which is normally avascular, produced significantly higher levels of median PDGF-AA, 202.62 pg/mL/g (range: 122.87-243.34 pg/mL/g), compared to the

synovium ($p<0.001$), 13.33 pg/mL/g (range: 7.42-32.82 pg/mL/g), and the ligamentum teres ($p=0.037$), 27.81 pg/mL/g (range: pg/mL/g) **(Figure 4-2P & Table 4-4)**.

Discussion:

The data from this study indicate that there was not a significant difference in the *ex vivo* metabolic responses of cartilage, synovium, ligamentum teres, and acetabular labrum tissues recovered from patients with end stage OA for a majority of the biomarkers analyzed. However, significant differences in the production of specific biomarkers among tissues may indicate unique responses and contributions to the inflammatory and degradative joint environment in end-stage OA of the hip.

In general, the data indicate that the metabolic responses of the ligamentum teres and the synovium were similar for the biomarkers analyzed in this study, as there were no significant differences observed between these two tissues. The metabolic responses of the labrum had similarities to the other three tissues, but the significant differences in biomarker production between tissues indicated that the labrum may be more similar to cartilage than synovium and ligamentum teres. The data also indicate that the metabolic responses of the cartilage and synovium are the least similar to each other, as all of the biomarkers identified as significantly different between tissues were significantly different between the cartilage and the synovium. Understanding these differences in tissue metabolic responses will help

in determining how each tissue may respond to the development of OA and identifying potential targets for intervention in future studies.

The production and regulation of MMPs and aggrecanases by joint tissues are an important factor in the development and progression of OA, as these degradative enzymes target the extracellular matrix of the tissue.[10,11] The data from this study identified potentially important differences in the production and regulation of degradative enzymes by the intra-articular tissues of the hip. The synovium, ligamentum teres, and labrum produced significantly higher levels of MMP-9 compared to the cartilage tissue, indicating they may be a significant source of MMP-9 in the hip joint during OA. Previous studies have indicated that the concentrations of MMP-9 in the serum and synovial fluid samples of OA patients were increased compared to health-hip controls.[12] Therefore, based on the MMP-9 production data in this study, the synovium, ligamentum teres, and labrum appear to be potential sources of higher MMP-9. Serum and synovial fluid concentrations observed in hip OA patients and previous research has correlated MMP-9 concentrations with rapid destructive OA of the hip[13,14]. MMP-9 is a gelatinase which can degrade many components of the extracellular matrix of orthopaedic tissues including collagens I, IV, V, VII, X, and XI, along with fibronectin, elastin, laminin, and vitronectin.[15–17] Additionally, it has been reported that MMP-9 may be a factor in stimulating apoptosis in hypertrophied chondrocytes and angiogenesis at the subchondral bone plate during OA.[18]

The cartilage tissue produced higher levels of MMP-3 and MMP-13, and the cartilage and labrum both produced significantly higher levels of MMP-7, compared

to the synovium and ligamentum teres. MMP-3 is a stromelysin which degrades collagens III, IV, V, IX, X, and XI along with proteoglycans, laminin, gelatins, fibronectin, and vitronectin.[16] The proteoglycans and fibronectin are of importance in reference to OA as extracellular matrix components that provide cartilage integrity and are degraded during the progression of OA.[19] In a study investigating meniscectomy patients, there were positive correlations for MMP-3 serum and synovial fluid concentrations and radiographic OA severity grade, indicating progression of knee osteoarthritis.[20] MMP-3 also has a well-defined role in activating other pro forms of MMPs.[21,22] In this study, higher levels of MMP-3 were produced by the cartilage, indicating that the cartilage may be a primary driver of imbalanced enzyme production and regulation in driving the development and progression of hip OA.

MMP-7 is a matrilysin which degrades collagen IV, proteoglycans, laminin, fibronectin, gelatins, elastin, and tenascin.[16] Again, the proteoglycans and fibronectin are of importance in reference to OA as they are extracellular matrix components that provide cartilage integrity. MMP-7 has been reported to be significantly increased in synovial fluid from knees with late-stage OA compared to healthy-knee controls, but not in early-stage knee OA.[23] In this study, the production of MMP-7 by the cartilage and the labrum was significantly higher than the synovium and LT, indicating that these tissues may be contributing to the shift towards degradation through MMP-7 production during OA. MMP-13 is a collagenase which degrades collagen I, II, III, VIII, and X along with gelatins and aggrecans.[16] Type II collagen and aggrecans are of importance in reference to OA

as type II collagen is the predominant collagen of the articular cartilage and aggrecan is the major proteoglycan of the articular cartilage.[24–27] A previous study indicated that the concentrations of MMP-13 in the synovial fluid of OA patients were increased in knees with the two most severe radiographic OA grades, 3 and 4, on the Kellegren-Lawrence scale.[28] In this study, MMP-13 production by the cartilage tissue, indicates that cartilage tissue may be the primary source of MMP-13 during the development and progression of hip OA. These results agree with previous research indicating that knee chondrocytes increase the production of MMP-3, MMP-7, and MMP-13 when cytokine stimulated.[29–32] Therefore, these data indicate that chondrocytes from both the knee and the hip increase the production of these degradative enzymes during OA development and production.

While MMPs can degrade aggrecan, the aggrecanases, ADAMTS4 and ADAMTS5, are significantly more efficient in targeting proteoglycans in the cartilage tissue during OA, and ADAMTS4 production can be upregulated by the inflammatory cytokines IL-1β and TNF-α.[33–38] Further, a previous study reported that the concentrations of ADAMTS4 in the synovial fluid of patients with knee OA were significantly higher than patients with a meniscal injury.[39] In the present study, ADAMTS4 production by the synovium and ligamentum teres was significantly higher than by the femoral head cartilage, indicating that these tissues may contribute to increased levels of ADAMTS4 in synovial fluid. Previous studies have indicated ADAMTS4 as a key enzyme in OA and is expressed by OA cartilage samples by *de novo* synthesis.[40,41] Another study reported that knee OA synovial fibroblasts increased ADAMTS4 expression when stimulated with cytokines.[42]

Based on this previous research, it was surprising that we noted only low levels of ADAMTS4 production from the osteoarthritic femoral head cartilage samples in the present study.

While there were not significant differences among tissues for production of the TIMPs analyzed in this study, the data from this study indicated differences among tissues' ability to regulate degradative enzymes. The level of MMP activity in synovial tissue cultures was significantly higher than the cartilage tissue cultures. Previous studies have shown that the synovial tissue produces active MMPs associated with radiographic signs of erosion, and the levels of active MMPs from OA synovial fibroblasts and OA synovial fluid samples were increased compared to healthy-joint samples.[43–45] While the data from this study do not indicate the mechanism responsible for the higher degradative enzyme activity in synovial tissue samples, it further supports the concept that the synovium is a significant contributor in the shift towards higher degradative enzyme activity in the osteoarthritic hip.

While OA is not classically considered an inflammatory disease, the presence of increased joint inflammation during the development and progression of OA is well established.[46–48] In agreement with previous studies on hip and knee OA samples, the data from this study indicated that the synovium is a significant source of inflammation in the hip.[49–51] The higher production of IL-6, GRO-α, and PGE_2 by the synovium relative to the cartilage may be indicative of their respective responses to inflammatory cytokine stimulation. Previous studies have indicated that the synovium increases the production of these biomarkers in response to

stimulation with IL-1β.[52,53] IL-6 is described to have a dual role in osteoarthritis as it induces pro-inflammatory mediators when it binds to the soluble IL-6 receptor, but increases anti-inflammatory factors when it binds to the membrane-bound receptor.[54] Increased levels of IL-6 in the serum and synovial fluid from OA patients has consistently correlated with incidence and severity of disease.[55–57] GRO-α induces apoptosis in the articular chondrocytes and is inducible by cytokines secreted by several cell types, including synovial monocytes and fibroblasts.[58–60] Interestingly, GRO-α has been indicated to contribute to the expression of IL-6 by synovial fibroblasts in OA joints.[61] PGE_2 has been described to decrease proteoglycan synthesis while enhancing degradation of aggrecan and type II collagen, and has been described to be upregulated by cytokines from a range of sources including the fibroblasts and synovial cells.[62–64] Further, a study found that synovial tissues from knee OA patients produced PGE_2 during *in vitro* experiments.[65] The data from the present study indicate that the synovium in OA hips may have similar inflammatory responses.

The higher production of NO by the cartilage relative to the synovium further indicates differential inflammatory responses among hip tissue types. Previous studies have indicated that cartilage increases the production of NO in response to cytokine stimulation.[66,67] NO has been shown to inhibit collagen and proteoglycan synthesis, enhance apoptosis, and inhibit adhesion to extracellular matrix when it is produced in excess during the pathogenesis of OA.[68,69] The results of the present study indicated that the cartilage from osteoarthritic hips has similar responses involving NO. Further studies are required to determine how the

levels of NO compare to healthy-hip controls to elucidate mechanisms and effects that may have clinical implications.

The higher production of PDGF-AA by the OA cartilage tissue relative to the other tissues may be indicative of an attempted repair response. Previous *in vivo* studies have found that PDGF promotes chondrocyte proliferation and migration, suppresses cell apoptosis, and increases proteoglycan production.[70–72] Previous studies have also indicated that PDGF-AA production by cartilage can be increased by IL-1β stimulation while simultaneously decreasing the PDGF receptor subunit to ensure paracrine effects of PDGF and not autocrine effects on the chondrocytes.[72–74] This indicates that the production of PDGF-AA by the cartilage may be as a paracrine signal to affect other tissues in the joint. Since a previous study found that PDGF-AA can recruit mesenchymal stem cells to the site of an osteochondral repair, it is possible that the higher production of PDGF-AA by the cartilage tissue in the hip is an attempt by the tissue to recruit these cells to the site of injury.[75] As such, higher levels of PDGF-AA in the synovial fluid of OA patients may be indicative of cartilage degradation.[76] The results from the present study suggest that PDGF-AA production by cartilage is associated with an attempted, albeit failed, repair response in osteoarthritic hips.

The higher production of GAG produced by the OA cartilage tissue relative to the ligamentum teres and the synovium may be indicative of tissue breakdown, synthesis, or the differential extracellular matrix composition of the tissue types. The assay, DMMB assay, utilized for this measurement is unable to differentiate between breakdown or synthesis. Studies have shown that with the progression of

OA there is decreased GAG in the articular cartilage and increased GAG in the synovial fluid of knees.[77,78] These mechanisms are likely similar for osteoarthritic hips based on the results of the present study.

As with any in vitro study, there are several limitations to consider when evaluating the data from this study and determining the clinical applicability of the findings. First, the tissues were removed from their native environment for explant culture such that biomarker production may be indicative of each tissue's response to culture and not specific to development of OA. Second, the samples were only cultured for 3 days, and therefore it is not clear if the differences in biomarker production by the tissue are representative of acute or chronic responses of the tissues. Third, the sample size for this study was relatively low for all tissue types, and therefore it is possible that the differences in tissue biomarker production observed in this study may not be generalizable. Fourth, there was no comparison to healthy-hip tissues, so it is not clear if the levels of biomarker production observed in this study represent a difference in tissue production due to OA, or just a difference in the basal production of these biomarkers by the tissues during ex vivo culture. Fifth, the structural changes in the tissues due to OA were not assessed, so relationships between tissue biomarker production and tissue changes related to OA could not be delineated. Sixth, not all tissue types were available for culturing from each sample that was received. Finally, relevant patient factors including age, sex, BMI, smoking status, medication use, and duration of symptoms were not accounted for in this study, so it is possible that differences in

tissue biomarker production were related to these factors and not the development of OA in the joint.

However, with these limitations in mind, the data from this study indicate significant differences in the production of inflammatory and degradative enzymes by key intra-articular tissues of the hip. Identifying tissue-specific responses can help elucidate mechanistic biomarkers for hip OA that may also be identified in synovial fluid, serum, and urine from patients. Delineating the source and roles of these biomarkers will aid in the development of early diagnosis, staging, and targeted treatment strategies aimed at optimizing the management of hip OA. Future studies with larger sample sizes that account for tissue degradation status and relevant patient factors are needed to translate this research towards understanding the pathophysiology of hip OA in clinical patients. Further understanding of tissue-specific roles in hip osteoarthritis may allow for novel biological targets for hip OA to increase patient-specific preventative and therapeutic treatment options.

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Sample ID	Cartilage	Synovium	Labrum	Ligamentum teres
1				X
2	X	X	X	X
3	X	X		
4	X	X	X	X
6	X	X		
8	X	X		X
9	X	X		X
10	X	X	X	X
12	X	X	X	X
13	X	X		X
15	X	X		
17	X	X	X	
19	X	X		
20	X	X	X	
22	X	X	X	
23	X	X	X	
24	X	X	X	
26	X	X	X	X
27	X	X		
28	X	X	X	
29	X	X		X
31	X	X	X	X
33	X			X
34	X	X		X
35	X	X	X	

Table 4-1: Tissue type collected for each sample

		Pairwise Comparison with Bonferroni Correction					
	Kruskal-Wallis significance	Cartilage vs. Labrum	Cartilage vs. Ligamentum Teres	Cartilage vs. Synovium	Labrum vs. Ligamentum Teres	Labrum vs. Synovium	Ligamentum Teres vs. Synovium
MMP-1	0.916						
MMP-2	0.726						
MMP-3	0.005 *	0.165	0.021 *	0.012 *	1.000	1.000	1.000
MMP-7	<0.001 *	1.000	<0.001 *	<0.001 *	0.012 *	0.008 *	1.000
MMP-8	0.090						
MMP-9	<0.001 *	0.006 *	<0.001 *	<0.001 *	1.000	1.000	1.000
MMP-13	<0.001 *	0.086	0.012 *	<0.001 *	1.000	1.000	1.000
MMP Activity	0.001 *	1.000	0.101	0.003 *	0.456	0.056	1.000
TIMP-1	0.142						
TIMP-2	0.724						
TIMP-4	0.042 *	1.000	0.266	0.062	1.000	0.726	1.000
ADAMTS-4	0.001 *	0.483	0.003 *	0.008 *	0.393	1.000	1.000
Media GAG	<0.001 *	0.078	0.002 *	<0.001 *	1.000	0.327	1.000
IL-2	0.524						
IL-4	0.171						
IL-6	0.019 *	1.000	0.335	0.016 *	1.000	0.576	1.000
IL-8	0.028 *	0.761	0.084	0.055	1.000	1.000	1.000
IL-10	0.533						
PGE2	0.001 *	1.000	0.993	0.004 *	0.562	0.006 *	0.728
Nitri Oxide	0.020 *	1.000	0.128	0.044 *	0.696	0.482	1.000
MCP-1	0.085						
MCP-3	0.872						
MIP-1α	0.328						
MIP-1β	0.066						
RANTES	0.294						
GRO-α	0.011 *	0.271	0.084	0.017 *	1.000	1.000	1.000
TNF-α	0.145						
VEGF	0.256						
PDGF-AA	<0.001 *	0.177	0.037 *	<0.001 *	1.000	0.572	1.000

Table 4-2: Kruskal-Wallis test p-values for biomarker comparisons by tissue type. P-values for post-hoc Dunn test pairwise comparisons using a Bonferroni correction provided where indicated. Significance of the two-sided result using $p<0.05$ indicated by *****.

	Femoral Head Cartilage	Acetabular Labrum	Synovium	Ligamentum Teres
MMP-1 (ng/mL/g)	155.02 (79.44-405.05)	260.14 (20.93-384.07)	225.01 (136.75-361.70)	210.40 (83.18-333.16)
MMP-2 (ng/mL/g)	267.64 (234.49-344.77)	271.41 (99.06-331.65)	369.91 (202.73-544.22)	314.83 (145.13-459.13)
MMP-3 (ng/mL/g)	9057 *****§ (7612-13901)	3682 (3212-4228)	2998 (2435-4430)	3444 (2419-4430)
MMP-7 (pg/mL/g)	2829 *§ (1825-5187)	2109 *§ (1230-3993)	500 (299.75-1748)	491 (338.07-1206)
MMP-8 (pg/mL/g)	1125 (333-2067)	1108 (538.50-1728.28)	1652 (1215-2303)	1652 (830-2224)
MMP-9 (pg/mL/g)	0 (0-123.83)	828 **^** (507.68-1360)	1276 **^** (994.41-1440)	1003 **^** (746.50-2012)
MMP-13 (ng/mL/g)	350 *§ (67.75-526.03)	16.10 (10.94-94.82)	4.73 (0.88-44.43)	6.29 (1.24-43.70)
MMP activity (ng/mL/g)	378.96 (183.10-639.12)	496.94 (215.36-637.54)	1386 **^** (641.69-3028)	1640.74 (442.47-2638)
ADAMTS-4 (pg/mL/g)	0 (0-29.19)	32.31 (19.79-111.48)	110.93 **^** (68.72-146.23)	163.54 **^** (155.09-191.90)
TIMP-1 (ng/mL/g)	305.37 (136.96-558.61)	139.52 (45.21-300.42)	224.97 (101.87-311.92)	397.75 (80.56-565.90)
TIMP-2 (ng/mL/g)	32.03 (19.31-48.46)	38.94 (14.66-55.03)	28.72 (8.99-47.69)	28.14 (12.06-87.95)
TIMP-4 (pg/mL/g)	706.38 (222.81-1409)	191.29 (141.67-1287)	71.68 (0-218.70)	128.35 (0-260.89)
Media GAG (mg/mL/g)	22.25 *§ (15.85-25.34)	9.44 (5.96-17.22)	4.95 (3.31-6.70)	7.72 (3.97-14.22)

Table 4-3: Median and interquartile range of biomarker concentration with *=significantly ($p<0.05$) increased over the synovium, **^**=significantly ($p<0.05$) increased over the femoral head cartilage, and **§**=significantly ($p<0.05$) increased over the ligamentum teres. Significance was determined by a Kruskal-Wallis test followed by a Dunn test pairwise comparison and Bonferroni correction for multiple comparisons

	Femoral Head Cartilage	Acetabular Labrum	Synovium	Ligamentum Teres
IL-2 (pg/mL/g)	0 (0-4.43)	0 (0-0.90)	0 (0-1.94)	0 (0-1.34)
IL-4 (pg/mL/g)	4.24 (0-14.90)	0 (0-2.85)	0 (0-3.08)	3.67 (0-4.70)
IL-6 (pg/mL/g)	126 (23.83-8701)	5344 (434.75-11745)	11461 ^ (7490-16295)	11362 (3699-15110)
IL-8 (pg/mL/g)	973 (240-12519)	12432 (6587-21632)	16508 (10639-21182)	15587 (10959-24665)
IL-10 (pg/mL/g)	61.86 (26.72-113.12)	130 (24.65-349.36)	106.27 (40.25-196.63)	46.72 (42.63-86.52)
PGE2 (pg/mL/g)	5160 (2042-7837)	4558 (1621-7248)	25050 ^† (11501-60864)	12104 (3998-27599)
Nitric Oxide (pg/mL/g)	0.67 * (0.19-1.44)	0.43 (0.25-0.53)	0.25 (0.09-0.33)	0.22 (0.12-0.47)
GRO-α (pg/mL/g)	246 (0-5043)	3987 (1640-8541)	4111 ^ (3084-7888)	4290 (2688-7629)
TNFα (pg/mL/g)	0 (0-106.55)	19.75 (0-49.88)	44.99 (11.11-126.19)	15.55 (8.61-39.89)
RANTES (pg/mL/g)	126.68 (76.17-292.17)	238.39 (108.75-751.95)	350.42 (186.08-637.78)	279.52 (150.42-616.30)
MIP-1α (pg/mL/g)	42.70 (0-567.08)	73.15 (0-874.24)	274.44 (72.53-2177)	124.66 (57.36-210.98)
MIP-1β (pg/mL/g)	21.60 (5.97-121.06)	117.16 (18.99-255.50)	222.56 (85.85-666.12)	279.52 (150.42-616.30)
MCP-1 (pg/mL/g)	17909 (9090-26993)	8841 (3172-17464)	11349 (6546-16608)	16514 (9805-21011)
MCP-3 (pg/mL/g)	81.85 (22.01-188.92)	113.66 (18.35-618.98)	111.90 (31.94-389.21)	59.83 (38.42-792.35)
VEGF (pg/mL/g)	2741 (1489-5265)	2777 (1104-6121)	1629 (782-2144)	2052 (1274-7777)
PDGF-AA (pg/mL/g)	202.62 §* (122.87-243.34)	45.97 (24.85-84.77)	13.33 (7.42-32.82)	27.81 (18.55-40.01)

Table 4-4: Median and interquartile range of inflammatory and anabolism related biomarker concentration with *=significantly ($p<0.05$) increased over the synovium, **^**=significantly ($p<0.05$) increased over the femoral head cartilage, **§**=significantly ($p<0.05$) increased over the ligamentum teres, and **†**=significantly ($p<0.05$) increased over the labrum. Significance was determined by a Kruskal-Wallis test followed by a Dunn test pairwise comparison and Bonferroni correction for multiple comparisons

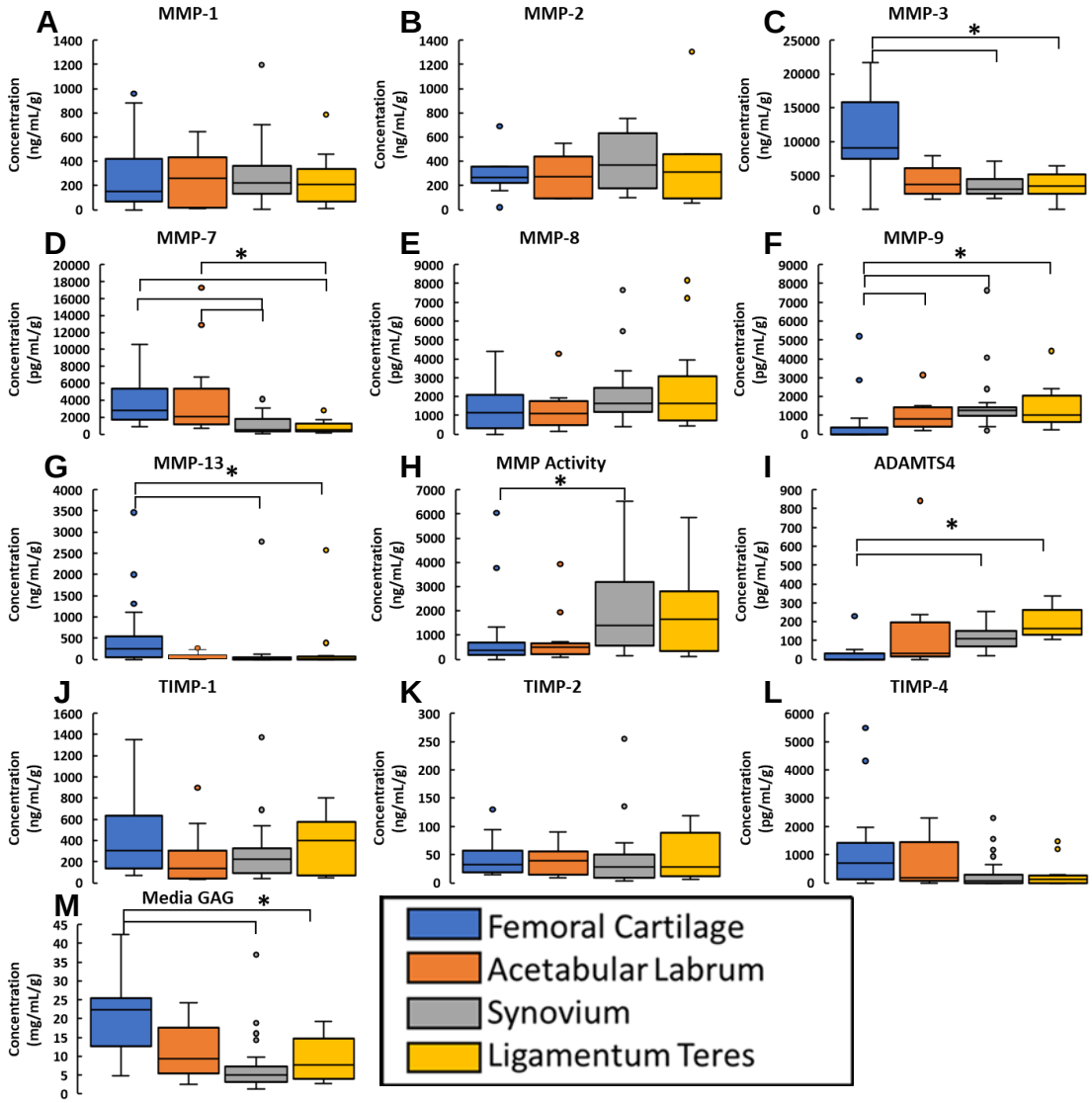

Figure 4-1: Degradative related differences between tissue types. Significant difference determined by Kruskal-Wallis followed by Dunn test pairwise comparison with Bonferroni correction for multiple comparisons (*=p<0.05)

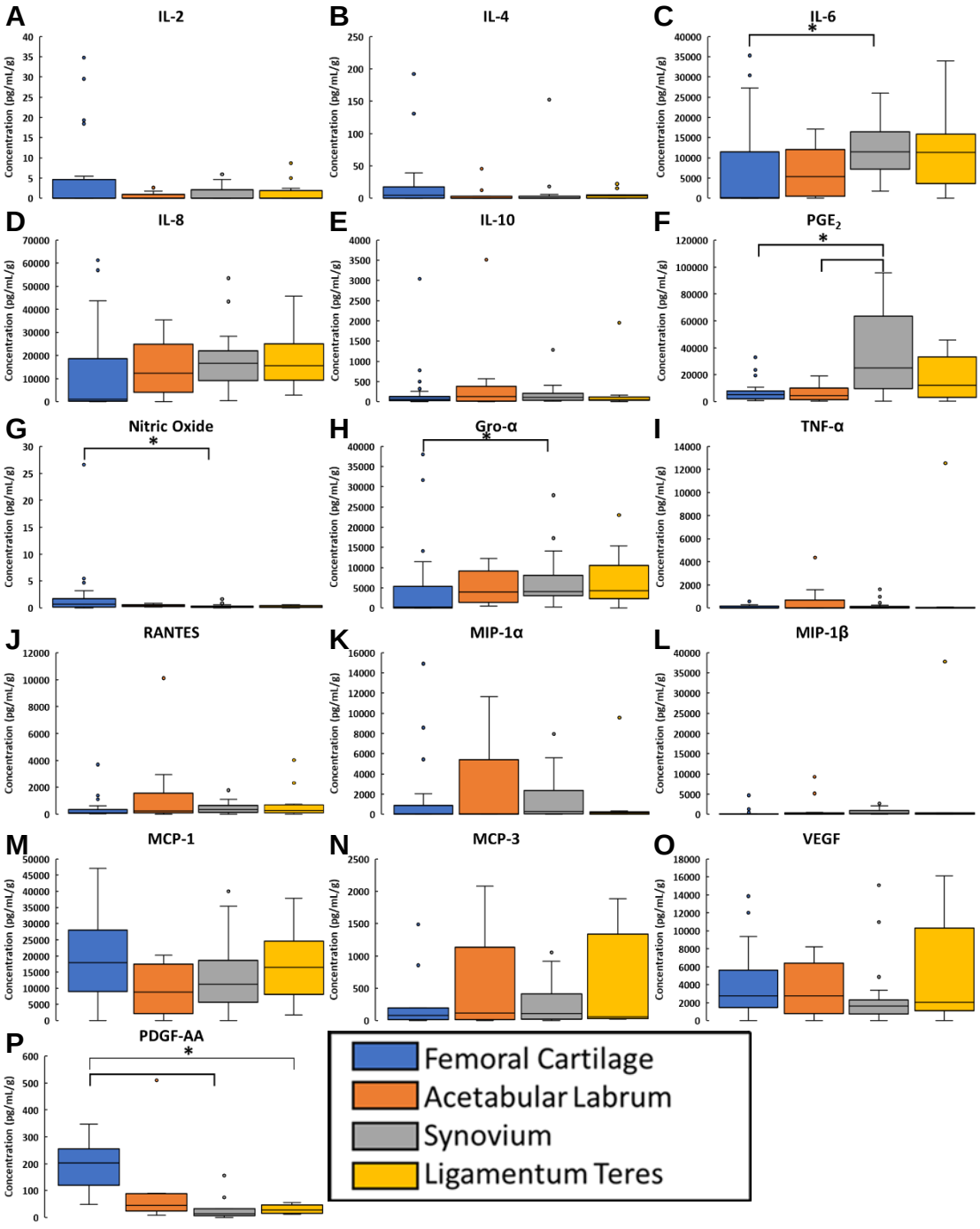

Figure 4-2: Inflammatory and anabolism related differences between tissue types. Significant difference determined by Kruskal-Wallis followed by Dunn test pairwise comparison with Bonferroni correction for multiple comparisons (*=p<0.05)

CHAPTER 5:

METABOLIC AND HISTOPATHOLOGIC EVALUATION OF OSTEOARTHRITIC ACETABULAR LABRUM

Research Objective and Hypothesis:

The acetabular labrum is thought to play important roles in hip joint biology and biomechanics.[1–4] It is also known that loss of labrum integrity and function contribute to the development and progression of hip osteoarthritis (OA).[5,6] Previous studies have indicated that acetabular labrum cells respond to inflammatory stimuli by increasing their expression of degradative proteins and other inflammatory proteins.[7] Another study reported that calcified acetabular labrum from osteoarthritic hips responded to inflammatory stimuli by increasing production of degradative enzymes and extracellular matrix.[8] While these studies have provided initial information, the acetabular labrum's metabolic responses that contribute to and correspond with disease mechanisms in hip OA have not been fully characterized.

Therefore, it is important to investigate the inflammatory and degradative metabolic responses of the acetabular labrum with respect to histopathologic severity scores in order to elucidate the potential biochemical contributions of labrum degeneration to the development of hip OA. As such, this study was designed to test the hypothesis that acetabular labrum explants from osteoarthritic hips would produce inflammatory and degradative biomarkers at levels that correspond with histopathologic severity scores. Elucidating these relationships

will help uncover mechanistic pathways for hip OA, allowing for development and validation of biomarkers for diagnostic, staging, and therapeutic applications.

Material and Methods:

Tissue Collection and Culture:

With IRB approval (#2016684) and informed patient consent, acetabular labrum tissues that would normally be discarded after surgery were recovered from patients (4 male, 4 female, mean age 57.8±8.69, mean BMI 32.3±6.08) undergoing total hip arthroplasty due to end stage OA. An approximate 10 mm wide full thickness labral explant was sterilely sectioned from each tissue sample using a scalpel blade and was cultured in 5 mL of supplemented Dulbecco's Modified Essential Medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA). DMEM was supplemented with 1mM sodium pyruvate, 2mM L-glutamine, 0.5mg/mL ascorbic acid, 1 x MEM N-E Amino Acid solution, 1% insulin transferrin selenium (ITS premix: BD Biosciences, Bedford, MA, USA), and 1x penicillin-streptomycin-amphotericin B (all components from Invitrogen Co., Carlsbad, CA, USA unless otherwise specified). Tissues were cultured for three days at 5% CO2, 37°C, and 95% humidity. On day 3 of culture, the media was collected and stored at -20ºC until used for biomarker testing outlined below. The wet weight of each tissue explant was recorded after culture.

Protein Analysis:

Multiplex Luminex assays were used to test day 3 culture media for matrix metalloprotease (MMP), MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13 using the High Performance Human MMP Magnetic Panel (R&D Systems, Minneapolis, MN, USA); tissue inhibitor of matrix metalloproteases (TIMP), TIMP-1, TIMP-2, TIMP-4 using the Human TIMP panel 2 (Millipore, Billerica, MA, USA); and interleukin (IL), IL-2, IL-4, IL-6, IL-8, IL-10, growth related oncogene (GRO)-α, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory proteins (MIP)-1α, MIP-1β, vascular endothelial growth factor (VEGF), regulated on activation normal T expressed and secreted (RANTES), and tumor necrosis factor (TNF)-α using the Human Cytokine Magnetic Panel (Millipore, Billerica, MA, USA). Media were also analyzed for Prostaglandin E_2 (PGE_2) using the Prostaglandin E_2 Express ELISA (Cayman Chemical Co., Ann Arbor, MI), nitric oxide using the 2,3-Diaminomaphthalene (DAN) assay[9], glycosaminoglycans (GAG) using the dimethylmethylene blue (DMMB) colorimetric assay[10], and MMP activity using the 520 MMP FRET Substrate XIV(Anaspec, Fremont, CA).[11] The concentration of each analyte was standardized to the tissue wet weight in grams to account for sample to sample variation in tissue size.

Histological Analysis:

A separate section of adjacent acetabular labrum and the associated acetabulum, collected the day of surgery, was placed in 10% neutral buffered formalin fixative for at least 24 hours and then placed in 10% ethylenediaminetetraacetic acid (EDTA) decalcifying solution until the bone was

softened (~10 days). After decalcification was complete, each specimen was routinely processed, sectioned (5 um), and stained with H&E and toluidine blue. The labrum was evaluated for degenerative changes by one board certified veterinary pathologist using both an in-house developed histopathologic scoring system and the published grading system from Ito et al. (2004), previously described.[12,13] Additionally, vascular proliferation (endothelial cell formation) was assessed using an in house developed scoring system on a scale from 0 to 3, with a score of 0 indicating no neovascularization and a score of 3 indicating marked neovascularization.

The in-house developed histopathologic scoring system evaluated labral tissues for (1) tears, (2) myxoid/mucinous degeneration, (3) cell proliferation, (4) inflammation, (5) cystic change, (6) calcification, (7) cell loss, and (8) granular matrix breakdown.[12,13] Each tissue was given a score of 0 (indicating no changes) to 3 (indicating severe changes) for each category, and the scores from all categories were summed to provide the total in-house labral histology score (0-24 points) for the tissue, with larger scores indicating greater pathology.

The labrum histologic grade, as described by Ito et al (2004)[13], is based on the dichotomous assessment of six morphologic abnormalities (1) the presence of dysplastic matrix, (2) hyperplasia or hypoplasia, (3) cysts, (4) hypercellularity or hypocellularity, (5) fibroblasts, and (6) the absence of large collagen fibers. If an abnormality is observed in the tissue section it is assigned one point, and sum of the score for all assessments is the histological grade for the tissue. Each tissue

can have a grade of 0-6 with greater scores indicating greater pathology. **(Figure 5-1)**

Statistical Analysis:

Mean biomarker concentrations regardless of histological score and for each histological scoring criteria are reported to gain an understanding of changes of biomarker concentration with histological scoring. Media biomarker data were standardized to the weight of the tissue explant. Trends were determined between the media biomarker concentrations and the in-house developed labral histological scoring system, the total summed histological grade, neovascularization, and the sub scores for the in-house scoring system.[13] Because the media biomarker data was not normally distributed and included a small number of samples (n=8), a Spearman's correlation was used to identify strong positive or negative ($r>0.6$ or $r< -0.6$) trends, rather than correlations, between all outcome measures. As such, only strong positive or negative ($r>0.6$ or $r< -0.6$) trends regardless of p-value are discussed. All statistical analyses were completed using R (v4.1.3) with RStudio and packages Hmisc and ggplot2 for all analyses.

Results:

Histological Measures and trends:

The data from the labral histological scoring system and the previously published labral histological grading system indicated the there was evidence of a range of degenerative changes observed in the labral tissues recovered from

patients undergoing total hip arthroplasty for OA. The total histological score ranged from 1-15 out of a possible 24, and the histological grade ranged from 2-6 out of a possible 6. While the strong positive trend (r=0.921) between the total histological score and the histological grade of the tissue indicates agreement between these two systems for assessing degenerative changes in the labrum, the data does indicate a potentially important difference in the range of degeneration assessed between these two methods. None of the labral tissues were given the maximum score using the scoring system, while two of the eight tissues were given the maximum score for the grading system. Therefore, it is possible that the wider range of the scoring system may be better able to categorize labral tissue degeneration for the assessment of changes in tissue metabolic responses related to tissue degeneration.

Using the in-house scoring system, the individual scores indicated that a range of myxoid/mucinous degeneration, cell proliferation, and granular matrix breakdown were observed in the labral tissues of this study **(Table 5-1)**, and a strong positive trend was observed between these scores (r=0.641-0.929), as well as to the total histological score (r=0.740-0.882) and histological grade (r=0.672-0.891) of the tissue **(Table 5-2)**. However, inflammatory cell infiltration and tissue calcification were not prominent features observed in the tissue utilized in this study, with no tissue sample getting a score over 1 for either assessment or no strong trend was observed for either of these scores to the other histological measures of the tissue. Some level of neovascularization was observed in most of the labral tissues used in this study, and a strong positive trend was observed

between the neovascularization score with the cellular loss score and the histological grade of the tissue, but not the total histological score for the tissue or the other tissue assessments of the in house labral histological score.

Trends among Histological Measures and Media Biomarkers:

Mean biomarker concentrations for each third of the scoring criteria and overall concentrations of the biomarkers regardless of histological grade were reported. **(Table 5-3, 5-4, 5-7)** The total histological score from the in-house scoring system and the previously described histological grade from Ito et al (2004)[13] shared a strong negative trend with the media concentration **(Table 5-5)** of IL-4, IL-6, IL-8, IL-10, MMP-1, MMP-8, and MMP-9 (r=-0.618 to -0.886), and a strong positive trend to media concentration of TIMP-4 (r=0.727 and 0.802). The only trend that was not shared between the scoring systems was the strong negative trend for level total MMP activity with the total in-house scoring system (r=-0.671). The consistency in the biomarker trends associated with the two histological assessments of labral degeneration associated with end stage hip OA indicates both systems are able to indicate labral pathologies that have trends with tissue metabolism.

Trends between the media biomarker concentrations and the individual scores of the in-house developed histological scoring system may be indicative of changes in tissue metabolism related to that specific feature. **(Table 5-5)** In agreement with the trends to the total histology score, there was a strong negative trend for the media concentration of IL-4, IL-6, IL-8, IL-10, MMP-1, MMP-8, and

MMP-9 with almost all the individual scores of the in-house histology scoring system (r=-0.664 to -0.964). There was a strong negative trend for the media concentration of TNF-α, MIP-1α, and MIP-1β with the individual histology scores for cell proliferation (r=-0.705 to -0.830), cellular loss (r=-0.705 to -0.788), myxoid degeneration (r=-0.729 to -0.870), and granular matrix breakdown (r=-0.708 to -0.727). There was a strong negative trend for the media concentration of MCP-1 and level of MMP activity with the individual histology scores for tissue tears (r=-0.805 and -0.753), myxoid degeneration (r=-0.717 and -0.704), cystic changes (r=-0.861 and -0.848), and granular matrix breakdown (r=-0.754 and -0.741). The media concentration of GRO-α had a strong negative trend with the individual histology scores for cellular proliferation, cellular loss, myxoid degeneration, and granular matrix breakdown (r=-0.701 to -0.865). There was a strong positive trend for the media concentration of MMP-2, MMP-3, and PGE2 with the cellular proliferation histology score (r=0.619 to 0.784). There was a strong positive trend for the media concentration of TIMP-4 with the individual histology scores for tissue tears, myxoid degeneration, cystic changes, cell loss, and granular matrix breakdown (r=0.730 to 0.825). There was a strong positive trend for the media concentration of TIMP-1, TIMP-2, and TIMP-4 for the neovascularity histology score (r=0.626 to 0.805). The trends between biomarkers and the assessment of specific factors related to labral degeneration related to end stage hip OA indicates IL-4, IL-6, IL-8, IL-10, MMP-1, MMP-8, and MMP-9 have similar shared negative trends with almost all scoring criteria. MMP-2 and MMP-3 have similar shared negative trends for some scoring criteria while similar positive trends for other

scoring criteria. GRO-α, MIP-1α, MIP-1β, TNF-α had similar shared negative trends. TIMP-1, TIMP-2, and VEGF all had only one positive trend >0.6 for neovascularization. TIMP-4 was unique in that it had positive trends to almost every scoring criterion reported.

Trends among Media Biomarkers:

Trends among the media biomarker concentrations produced by the tissue may also indicate shared production regulation pathways that could be targets for the treatment. **(Table 5-6)** There were shared positive trends between IL-4, IL-6, IL-8, and IL-10 (r=0.764 to 0.976). There were similar positive trends of IL-6, IL-8, and IL-10 to GRO-α, MIP-1α, MIP-1β, TNF-α, MMP-1, and MMP-9 (r=0.714 to 0.922) while a negative trend to TIMP-4 (r=-0.714 to -0.857). There were shared positive trends between MIP-1α to GRO-α (r=0.970) and TNF-α to MIP-1β (r=0.898). There were shared negative trends of MMP-2 and MMP-3 to GRO-α, MIP-1α, and TNF-α (r=-0.690 to -0.905). There were shared positive trends of MIP-1β and TNF-α to GRO-α and MIP-1α (r=0.762 to 0.922). There were negative trends between IL-2 to MMP-13 (r=-0.799), IL-8 to MMP-3 (r=-0.762), and MIP-1β to MMP-3 (r=-0.905). There were positive trends between IL-4 to MMP-8 and MMP-9 (r=0.736 and 0.791), PGE_2 to MMP-13 (r=0.850), MCP-1 to MMP-8 (r=0.786), RANTES to MMP-7 (r=0.833), and VEGF to MMP-7 (r=0.786). There were negative trends between TIMP-4 to IL-4 and TNF-α (r=-0.791 and -0.731). There were positive trends between total MMP activity with IL-10 and MCP-1 (r=0.786 and 0.738) along with GAG released to the media and nitric oxide

(r=0.714). There were positive trends between MMP-1 to MMP-9 (r=0.905), MMP-2 to MMP-3 (r=0.881), and MMP-2 to MMP-13 (r=0.714). There was a negative trend of MMP-9 to TIMP-4 (r=-0.857). There was a positive trend between TIMP-1 and TIMP-2 (r=0.922).

Discussion:

The data from this study indicate trends for correspondence between inflammatory and degradative metabolic responses of acetabular labrum explants in culture and the severity of labral degeneration as indicated by histopathologic severity score and grade. Specifically, there was a decrease in the production of MMPs, cytokines, and chemokines as labral degeneration became more severe. Therefore, earlier stages of labral pathology may be associated with more robust pro-degradative and pro-inflammatory responses. Further, there appeared to be an anti-degradative response by the labrum during later stages of tissue degeneration based on the positive trend for TIMP-4 production, and negative trend for MMP activity, with worsening histopathological severity measures.

Previous studies demonstrated that degenerative acetabular labrum from osteoarthritic hips significantly increased the production of MMP-1, MMP-9, and IL-6 in cell culture with IL-1 stimulation, while MMP-13 and IL-6 production increased with lipopolysaccharide (LPS) stimulation.[7,8] In the present study, the production of MMP-1, MMP-9, and IL-6 all had negative trends corresponding to severity of labral tissue degeneration. While our study focused on histological measures and their relation to biomarkers, previous studies focused on biomarker

production following stimulation with various cytokines such that it is difficult to compare among studies.

While there are few studies assessing the metabolic responses of the acetabular labrum a similar structure in the knee, the meniscus, has been more thoroughly evaluated.[14,15] Acute meniscal tears were associated with increases in MMP-9 and TNF-α present in the synovial fluid.[16] In the current study, similar increases in MMP-9 and TNF-α were found with less severe labral tears, indicating a similar relationship between these metabolites. When menisci were stimulated with IL-10, there was a decrease in cell-related death after subsequent TNF-α stimulation compared to TNF-α stimulation alone, indicating a protective effect of IL-10.[17] In this study, a positive trend between IL-10 and TNF-α was observed indicating that IL-10 may be responding to increases in TNF-α to protect the acetabular labrum.

There were no positive trends seen with MMP-13 and labral degeneration with an $r>0.6$. This was unexpected as a previous study indicated that MMP-13 production is increased at later stages of OA.[18,19] This result could be due to sample origin, as Xin et al. (2021) used serum and tissue samples from human knees while Lim et al. (2014) analyzed knee OA following meniscus destabilization in murine tissue. The labrum also may not be the largest producer of MMP-13 in an osteoarthritic hip joint.

In meniscal studies, gene expression has been analyzed and compared with traumatic tears and chronic degenerative tears. Results from these studies indicate IL-8 was significantly increased with traumatic tears indicating an early

cytokine response to tissue damage.[20] Similarly, there was a positive trend in IL-8 with early stage labral degeneration in our study. MIP-1α also has been implicated as a potential early marker of degeneration based on a previous *in vivo* study, which analyzed the synovial fluid in knees two weeks after an injection with monosodium urate crystals.[21] MIP-1α showed a negative trend to several of the labrum histological measures in our study indicating it may be associated early response to labral damage in hip OA.

In human meniscal samples of early- and late-stage OA, IL-6 was present in higher concentrations in late-stage OA.[22] In contrast, our results showed a negative trend between IL-6 and histological severity grade, which may indicate the meniscus and labrum have differing responses to degeneration with respect to the production of IL-6.

Previous research has indicated that as OA progresses, there is a significant decrease in the concentrations of TIMP-1 in the serum of humans and dogs.[23,24] Another study found that TIMP-1 was decreased in rabbits with PCL transection compared to healthy-knee controls.[25] One study evaluated MMP and TIMP expression of damaged labral tissue from patients with FAI and found the expressions of MMP-1 and MMP-2 were increased while the expression of TIMP-1 was reduced, when compared to normal labrum.[26] Interestingly, our results only found a positive trend for TIMP-1 production and neovascularization. There were no trends with the other histological severity scores/grades of the labrum. It is important to note that our study did not include any healthy-hip samples, which may still have an increased production of TIMP-1 over OA samples. TIMP-4 gene

expression has been shown to decrease in the presence of OA in studies analyzing canine and human cartilage from healthy and OA joints.[27,28] In the knee, the meniscus appears to be a primary source of TIMP-4 with the highest production occurring in late-stage OA.[29] In our study, there was a positive trend observed between TIMP-4 with histological severity scores/grades, indicating that TIMP-4 production by labrum may be an anti-degradative response in an attempt to slow the progression of labral tissue degradation.

In the present study, there were numerous positive trends observed between the production of inflammation related biomarkers and degradative enzymes by the labral tissue. In human OA cartilage, IL-4 has been reported to decrease the release of MMP-13 and RANTES after IL-1β stimulation indicating a potential anti-inflammatory and anti-catabolic role for IL-4.[30] The positive trend observed between IL-4 and other inflammatory biomarkers (IL-6, IL-8, and IL-10) and degradative enzymes (MMP-1, MMP-8, and MMP-9) observed in the present study may indicate that the labrum responding to the these biomarker stimuli by attempting to increasing the production IL-4. Similar positive trends between these proteins and IL-4 have been described in previous cell culture studies,[31,32] further supporting the concept that the trends observed for the production of IL-4 may be indicative of an attempt by the labral tissue to reduce inflammation and degradation, but further targeted research would be needed to confirm or deny this hypothesis.

As described previously, TIMP production is increased in an attempt to regulate the activity of MMPs and ADAMTs and reduce tissue breakdown.[33–36] Our

study indicated there was a positive trend between TIMP-1 and TIMP-2. The production of these two important regulators of degradative enzyme activity appear to share similar regulatory mechanisms in the labrum. Surprisingly, the production of TIMP-1 or TIMP-2 did not have a positive trend to the other anti-degradative proteins or a negative trend to degradative MMPs or cytokines as noted in the literature.[37–40] This may indicate the labrum does not change production of TIMP-1 or TIMP-2 in response to degradative and inflammatory proteins once it has progressed to degeneration in an osteoarthritic hip.

This study has several limitations that should be considered when assessing the conclusions and clinical applicability of the data. First, this is an *in vitro* study, and the tissues were removed from their native environment. The biomarkers being released into the media may indicate responses to culture and not to acetabular labral degeneration related to OA development in the hip. Second, this study used a 3-day culture period such that the findings may not be representative of the complex regulation of protein production that occurs during the course of a chronic disease like osteoarthritis. Third, only correlations of proteins released during culture were assessed. Therefore, the data can only suggest an interaction between tissue degradation and protein production and may not be reflective of a clinically significant increase in protein production associated with labral tissue degradation during OA. Fourth, all tissue samples come from individuals with hip osteoarthritis who are receiving total hip replacements, therefore these samples are representative of end-stage OA and may not be representative of tissue across the spectrum of OA progression in the hip. Fifth,

patient demographic factors were not accounted for, which may have significant effects on metabolic responses. Finally, the sample size for this study was small, limiting definitive conclusions regarding correlations such that only trends were reported. Further analysis with increased sample size is needed for correlations to represent the broader hip OA patient population.

With these limitations in mind, the data from this study indicate that acetabular labrum explants from osteoarthritic hips produce inflammatory and degradative biomarkers at levels that show trends with histopathologic severity scores. The findings suggest that the labrum produces inflammatory and degradative related factors in the end-stage of OA in the hip. Further, as degradation of the labrum increases, the tissue increases the production of TIMP-4, which may be associated with an attempt to counteract tissue degradation through the regulation of degradative enzyme activity. Several previous studies have indicated a relationship between increased joint inflammation and increased degradative enzyme production and activity during OA progression and development.[41–46] These interactions may be indicative of shared mechanisms of regulation that could be targeted to reduce degradative and inflammatory responses that occur during the development and progression of hip OA. Further studies aimed at assessing tissues from a wider spectrum of labrum and joint pathology and accounting for patient demographic factors are likely needed to elucidate the effects of acetabular labral degradation during the development and progression of hip OA.

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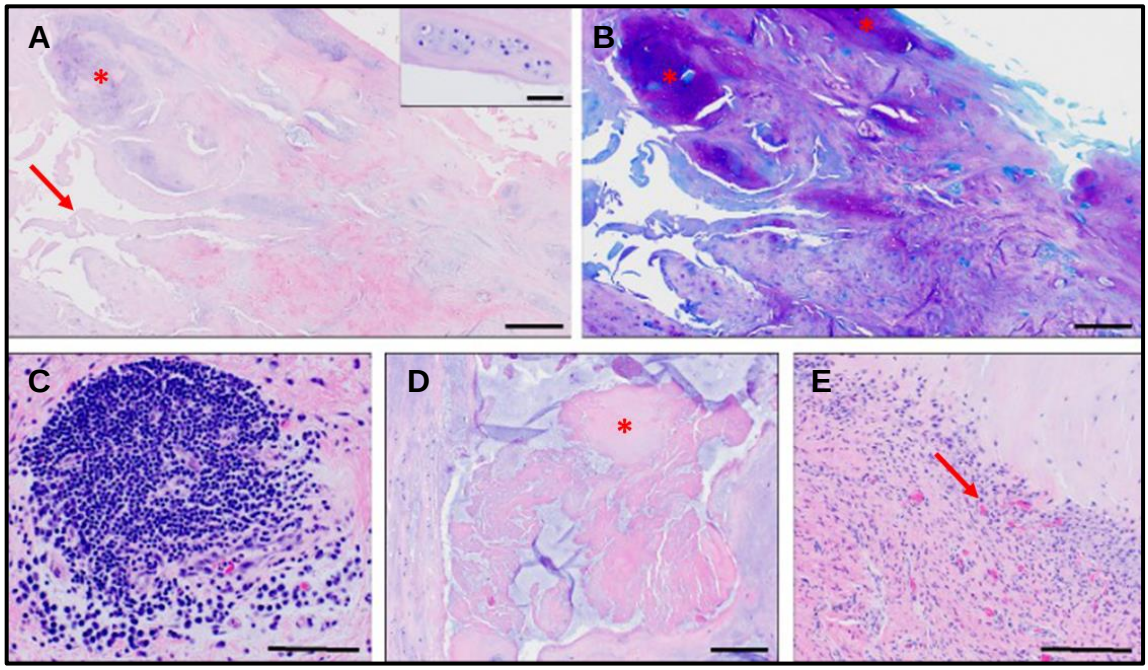

Figure 5-1: Representative histological changes observed in degenerative acetabular labra: a) Deep tears (arrow), cell proliferation (inset with scale bar = 50 μm), myxoid changes (*), cystic changes (arrowhead), and cellular loss (H&E; scale bar = 500 μm); b) Prominent myxoid changes (*; Toluidine blue; scale bar = 500 μm); c) Inflammatory response (H&E; scale bar = 100 μm); d) Granular matrix breakdown (*) with myxoid change (H&E; scale bar = 200 μm); d) Neovascularization (arrow; H&E; scale bar = 200 μm).

Sample	Tears	Myxoid	Proliferation	Inflammation	Cystic	Calcification	Loss	Breakdown	Total	Neovascularization	Grade	Sex	Age	BMI
1	3	3	3	0	3	0	2	3	14	1	5	M	63.9	29
2	0	1	2	0	0	0	1	0	4	1	3	F	49.26	30.6
3	3	3	2	0	3	0	2	2	12	1	5	M	40.58	36.1
4	2	2	2	0	0	0	2	1	9	3	4	F	59.44	39
5	1	0	1	1	0	1	0	0	4	1	2	M	57.92	42
6	0	0	1	0	0	0	0	0	1	0	2	F	57.92	31.9
7	3	2	2	0	2	1	2	1	13	2	6	F	62.62	22
8	3	2	2	0	3	1	2	2	15	2	6	M	70.91	27.6
Mean	1.88	1.63	1.88	0.13	1.38	0.38	1.38	1.13	9.00	1.38	4.13		57.82	32.28
Standard Deviation	1.27	1.11	0.60	0.33	1.41	0.48	0.86	1.05	5.00	0.86	1.54		8.69	6.08

Table 5-1: Histological score and demographic factor for each respective sample

	Tears	Myxoid	Proliferation	Inflammation	Cystic	Calcification	Loss	Breakdown	Total	Neovascularization
Myxoid	**0.842**									
Proliferation	**0.641**	**0.856**								
Inflammation	-0.265	-0.514	-0.571							
Cystic	**0.895**	**0.826**	**0.648**	-0.361						
Calcification	0.302	-0.234	-0.260	0.488	0.123					
Loss	**0.863**	**0.899**	**0.810**	-0.571	**0.723**	0.000				
Breakdown	**0.863**	**0.929**	**0.799**	-0.428	**0.913**	-0.058	**0.856**			
Total	**0.863**	**0.764**	**0.740**	-0.332	**0.866**	0.340	**0.850**	**0.882**		
Neovascularization	0.473	0.338	0.376	-0.177	0.140	0.363	**0.686**	0.325	0.552	
Grade	**0.891**	**0.730**	**0.672**	-0.504	**0.797**	0.344	**0.882**	**0.761**	**0.921**	**0.605**

Table 5-2: Trends among histological measures. r>0.6 or <-0.6 indicated by bold numbers

	IL-2 (pg/mL/g)	IL-4 (pg/mL/g)	IL-6 (pg/mL/g)	IL-8 (pg/mL/g)	IL-10 (pg/mL/g)	NO (pg/mL/g)	PGE_2 (pg/mL/g)	GRO-α (pg/mL/g)	MCP-1 (pg/mL/g)	MIP-1α (pg/mL/g)	MIP-1β (pg/mL/g)	RANTES (pg/mL/g)	TNF-α (pg/mL/g)	VEGF (pg/mL/g)
Tears														
0-1 (n=3)	0.88	3.57	13584.11	21815.42	258.02	0.29	1893.72	7975.91	17604.06	6684.66	1841.08	1179.38	984.60	2577.70
2 (n=1)	0.90	0.00	3186.81	6587.41	75.56	0.39	4145.89	1639.88	17906.09	36.38	4.50	232.05	0.00	6547.45
3 (n=4)	0.65	0.00	2637.77	5886.34	42.89	0.26	5386.55	2433.69	5807.05	177.07	55.69	152.84	9.06	6380.53
Myxoid														
0-1 (n=3)	0.88	3.57	13584.11	21815.42	258.02	0.29	1893.72	7975.91	17604.06	6684.66	1841.08	1179.38	984.60	2577.70
2 (n=3)	0.30	0.00	4424.05	9225.47	78.64	0.35	7802.10	3089.39	12274.97	248.23	64.89	169.82	11.53	8517.04
3 (n=2)	1.30	0.00	232.88	1228.19	5.60	0.19	1142.89	1053.23	2154.69	0.00	16.30	166.96	0.83	3259.22
Proliferation														
0-1 (n=2)	1.32	2.62	14694.99	25124.04	304.50	0.37	0.00	11025.52	15900.35	9998.05	2700.79	1485.51	1467.60	3121.62
2 (n=5)	0.18	1.09	5015.03	8863.43	80.98	0.28	5962.48	2397.08	11794.81	160.51	67.80	234.42	10.63	5526.42
3 (n=1)	2.61	0.00	25.11	1013.79	7.29	0.16	1560.80	1265.95	3171.74	0.00	9.92	238.39	1.67	5927.28
Cystic														
0-1 (n=4)	0.88	2.68	10984.79	18008.42	212.41	0.31	2456.76	6391.90	17679.57	5022.59	1381.94	942.54	738.45	3570.14
2 (n=1)	0.00	0.00	7501.39	12432.13	130.01	0.20	18966.71	5536.84	8841.00	635.15	171.18	108.75	32.54	2032.13
3 (n=3)	0.87	0.00	1016.57	3704.41	13.85	0.28	859.83	1399.31	4795.73	24.38	17.20	167.53	1.24	7829.99
Loss														
0-1 (n=3)	0.88	3.57	13584.11	21815.42	258.02	0.29	1893.72	7975.91	17604.06	6684.66	1841.08	1179.38	984.60	2577.70
2 (n=5)	0.70	0.00	2747.58	6026.56	49.43	0.29	5138.41	2274.93	8226.86	148.94	45.45	168.68	7.25	6413.91
3 (n=0)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Breakdown														
0-1 (n=5)	0.71	2.14	10288.11	16893.16	195.93	0.29	5758.75	6220.89	15911.85	4145.11	1139.79	775.79	597.27	3262.54
2 (n=2)	0.00	0.00	1512.30	5049.72	17.13	0.34	509.34	1465.99	5607.73	36.57	20.83	132.10	1.02	8781.35
3 (n=1)	2.61	0.00	25.11	1013.79	7.29	0.16	1560.80	1265.95	3171.74	0.00	9.92	238.39	1.67	5927.28
Total														
0-6 (n=3)	0.88	3.57	13584.11	21815.42	258.02	0.29	1893.72	7975.91	17604.06	6684.66	1841.08	1179.38	984.60	2577.70
7-12 (n=2)	0.45	0.00	1813.73	4015.00	39.73	0.30	2435.43	1240.20	9521.87	18.19	13.59	163.78	0.00	3569.30
13-18 (n=3)	0.87	0.00	3370.15	7367.59	55.89	0.28	6940.40	2964.75	7363.52	236.10	66.70	171.94	12.08	8310.32
19-24 (n=0)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Endothelial														
0-1 (n=5)	1.05	2.14	8243.62	13580.53	157.05	0.25	1593.39	5206.84	11424.31	4010.80	1111.17	774.41	591.09	2850.30
2 (n=2)	0.00	0.00	5042.67	10544.50	80.18	0.33	9630.20	3814.15	9459.41	354.15	95.08	138.71	17.29	9501.84
3 (n=1)	0.90	0.00	3186.81	6587.41	75.56	0.39	4145.89	1639.88	17906.09	36.38	4.50	232.05	0.00	6547.45
Grade														
0-2 (n=2)	1.32	2.62	14694.99	25124.04	304.50	0.37	0.00	11025.52	15900.35	9998.05	2700.79	1485.51	1467.60	3121.62
3-4 (n=2)	0.45	2.73	7274.58	10892.80	120.31	0.25	4913.52	1758.29	19458.79	47.14	63.09	399.57	9.29	4018.65
5-6 (n=4)	0.65	0.00	2637.77	5886.34	42.89	0.26	5386.55	2433.69	5807.05	177.07	55.69	152.84	9.06	6380.53

Table 5-3: Inflammatory, immune cell signaling, and anabolism mean biomarker concentration for histological scoring

	MMP-1 (ng/mL/g)	MMP-2 (ng/mL/g)	MMP-3 (ng/mL/g)	MMP-7 (pg/mL/g)	MMP-8 (pg/mL/g)	MMP-9 (pg/mL/g)	MMP-13 (ng/mL/g)	TIMP-1 (ng/mL/g)	TIMP-2 (ng/mL/g)	TIMP-4 (pg/mL/g)	MMP Act (ng/mL/g)	MGAG (mg/mL/g)
Tears												
0-1 (n=3)	326.92	460.08	1050.03	5459.37	1961.23	1831.61	129.10	161.38	34.85	91.81	941.67	12.85
2 (n=1)	270.27	1206.91	4949.25	2953.05	1796.54	766.47	41.81	563.04	83.88	1286.67	717.95	23.30
3 (n=4)	73.61	81.33	1225.28	2473.22	500.45	322.67	104.31	169.81	34.03	1373.93	232.71	10.22
Myxoid												
0-1 (n=3)	326.92	460.08	1050.03	5459.37	1961.23	1831.61	129.10	161.38	34.85	91.81	941.67	12.85
2 (n=3)	177.40	446.85	1709.91	2574.00	889.19	495.06	110.18	387.26	65.18	1299.13	481.72	15.67
3 (n=2)	16.25	95.85	2360.32	2561.97	565.38	285.99	64.26	40.26	12.24	1442.50	101.82	8.58
Proliferation												
0-1 (n=2)	385.18	37.28	50.35	6798.54	1829.66	2329.76	0.23	143.17	41.79	73.54	1128.45	16.47
2 (n=5)	152.70	549.06	1936.05	2326.85	1176.87	514.32	166.55	278.97	45.27	1130.98	417.16	12.76
3 (n=1)	11.57	92.64	3219.53	3992.68	138.37	320.86	13.16	45.21	14.62	1255.82	131.13	5.96
Cystic												
0-1 (n=4)	312.75	646.79	2024.83	4832.79	1920.06	1565.32	107.28	261.79	47.10	390.53	885.74	15.46
2 (n=1)	252.17	65.51	88.47	1706.37	315.99	507.68	272.62	300.42	56.63	387.08	514.91	14.28
3 (n=3)	14.09	86.61	1604.22	2728.84	561.93	261.00	48.21	126.28	26.50	1702.88	138.65	8.87
Loss												
0-1 (n=3)	326.92	460.08	1050.03	5459.37	1961.23	1831.61	129.10	161.38	34.85	91.81	941.67	12.85
2 (n=5)	112.94	306.45	1970.08	2569.18	759.67	411.43	91.81	248.46	44.00	1356.48	329.76	12.84
3 (n=0)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Breakdown												
0-1 (n=5)	300.64	530.53	1637.56	4207.51	1599.25	1353.80	140.35	269.52	49.01	389.84	811.57	15.22
2 (n=2)	15.34	83.60	796.57	2096.91	773.71	231.07	65.73	166.81	32.44	1926.41	142.40	10.32
3 (n=1)	11.57	92.64	3219.53	3992.68	138.37	320.86	13.16	45.21	14.62	1255.82	131.13	5.96
Total												
0-6 (n=3)	326.92	460.08	1050.03	5459.37	1961.23	1831.61	129.10	161.38	34.85	91.81	941.67	12.85
7-12 (n=2)	145.60	652.99	3225.19	2042.15	1394.47	508.80	78.58	299.17	46.87	1457.92	395.23	17.25
13-18 (n=3)	91.17	75.42	1133.34	2920.54	336.46	346.52	100.63	214.65	42.09	1288.85	286.12	9.89
19-24 (n=0)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Endothelial												
0-1 (n=5)	202.65	314.39	1574.15	4300.41	1402.89	1213.36	103.16	112.93	25.80	632.08	605.73	11.14
2 (n=2)	130.96	66.82	90.24	2384.47	435.51	359.35	144.36	299.37	55.83	1305.36	363.61	11.86
3 (n=1)	270.27	1206.91	4949.25	2953.05	1796.54	766.47	41.81	563.04	83.88	1286.67	717.95	23.30
Grade												
0-2 (n=2)	385.18	37.28	50.35	6798.54	1829.66	2329.76	0.23	143.17	41.79	73.54	1128.45	16.47
3-4 (n=2)	240.33	1256.30	3999.32	2867.04	2010.47	800.89	214.32	380.41	52.41	707.51	643.03	14.44
5-6 (n=4)	73.61	81.33	1225.28	2473.22	500.45	322.67	104.31	169.81	34.03	1373.93	232.71	10.22

Table 5-4: Degradative related biomarker concentration for histological scoring.

	IL-2	IL-4	IL-6	IL-8	IL-10	NO	PGE_2	GRO-α	MCP-1	MIP-1α	MIP-1β	RANTES	TNF-α
Tears	-0.204	**-0.878**	**-0.792**	**-0.728**	**-0.830**	-0.026	0.238	-0.511	**-0.805**	-0.475	-0.472	-0.217	-0.507
Myxoid	-0.165	**-0.807**	**-0.964**	**-0.964**	**-0.964**	-0.358	0.398	**-0.865**	**-0.717**	**-0.870**	**-0.729**	-0.185	**-0.826**
Proliferation	-0.103	-0.583	**-0.866**	**-0.866**	**-0.784**	-0.591	**0.622**	**-0.784**	-0.399	**-0.830**	**-0.784**	0.124	**-0.705**
Inflammation	0.439	0.472	0.577	0.577	0.412	0.577	-0.498	0.412	0.247	0.581	0.577	0.577	0.581
Cystic	-0.201	**-0.717**	**-0.848**	**-0.730**	**-0.848**	-0.156	0.052	-0.548	**-0.861**	-0.551	-0.430	-0.274	-0.492
Calcification	-0.180	-0.065	0.282	0.394	0.169	0.507	-0.113	0.507	-0.056	**0.624**	0.394	0.169	0.510
Loss	-0.351	**-0.898**	**-0.866**	**-0.866**	**-0.866**	-0.206	0.539	**-0.701**	-0.536	**-0.705**	**-0.784**	-0.206	**-0.788**
Breakdown	-0.020	**-0.849**	**-0.964**	**-0.914**	**-0.927**	-0.148	0.162	**-0.704**	**-0.754**	**-0.727**	**-0.717**	-0.173	**-0.708**
Total	-0.223	**-0.782**	**-0.790**	**-0.695**	**-0.778**	-0.060	0.283	-0.467	-0.599	-0.458	-0.587	-0.036	-0.482
Neovascularization	-0.367	-0.571	-0.294	-0.332	-0.358	0.217	0.520	-0.243	0.102	-0.180	-0.562	0.128	-0.430
Grade	-0.510	**-0.792**	**-0.703**	**-0.618**	**-0.703**	-0.182	0.476	-0.400	-0.594	-0.402	-0.485	-0.267	-0.457

	VEGF	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-13	TIMP-1	TIMP-2	TIMP-4	MMP Act	MGAG
Tears	0.345	**-0.664**	0.026	0.217	0.166	**-0.907**	**-0.856**	0.153	-0.051	-0.051	**0.805**	**-0.753**	0.166
Myxoid	0.124	**-0.741**	0.469	**0.630**	0.025	**-0.679**	**-0.840**	0.346	-0.222	-0.296	**0.741**	**-0.704**	-0.124
Proliferation	0.234	**-0.784**	**0.619**	**0.784**	0.206	-0.577	**-0.701**	0.454	-0.014	-0.206	0.522	-0.495	-0.399
Inflammation	0.247	0.577	-0.577	-0.577	0.577	0.082	0.577	-0.577	-0.082	-0.082	-0.247	-0.082	0.577
Cystic	0.143	**-0.848**	0.078	0.196	0.078	**-0.809**	**-0.913**	0.104	-0.365	-0.365	**0.730**	**-0.848**	-0.196
Calcification	0.507	0.056	**-0.620**	**-0.620**	0.394	-0.394	-0.056	-0.169	0.394	0.394	0.169	-0.169	0.507
Loss	0.371	**-0.701**	0.454	**0.619**	0.041	**-0.660**	**-0.866**	0.454	0.206	0.206	**0.825**	-0.495	0.041
Breakdown	0.309	**-0.803**	0.247	0.494	0.198	**-0.803**	**-0.889**	0.074	-0.222	-0.222	**0.803**	**-0.741**	-0.124
Total	0.539	**-0.814**	0.132	0.323	0.335	**-0.850**	**-0.886**	0.180	0.108	0.036	**0.802**	**-0.671**	-0.036
Neovascularization	**0.741**	-0.217	0.294	0.409	0.281	-0.294	-0.409	0.370	**0.805**	**0.677**	**0.626**	-0.013	0.460
Grade	0.339	**-0.752**	0.182	0.267	0.012	**-0.752**	**-0.885**	0.449	0.230	0.158	**0.727**	-0.521	-0.024

Table 5-5: Trends among histological measures and media protein concentrations. r>0.6 or <-0.6 indicated by bold numbers

	IL-2	IL-4	IL-6	IL-8	IL-10	NO	PGE_2	GRO-α	MCP-1	MIP-1α	MIP-1β	RANTES	TNF-α	VEGF
IL-4	0.131													
IL-6	0.101	**0.791**												
IL-8	0.038	**0.791**	**0.976**											
IL-10	0.216	**0.764**	0.952	**0.929**										
NO	0.190	-0.055	0.310	0.333	0.190									
PGE_2	-0.447	-0.233	-0.275	-0.347	-0.252	**-0.683**								
GRO-α	0.152	0.491	**0.833**	**0.881**	**0.882**	0.405	-0.395							
MCP-1	0..025	**0.682**	**0.667**	0.595	**0.667**	0.143	0.060	0.381						
MIP-1α	0.108	0.521	**0.874**	**0.922**	**0.850**	0.503	0.410	**0.970**	0.395					
MIP-1β	0.051	**0.655**	**0.810**	**0.857**	**0.738**	0.167	-0.371	**0.762**	0.143	**0.814**				
RANTES	0.330	0.436	0.190	0.167	0.119	0.024	0.108	-0.071	0.500	0.048	-0.024			
TNF-α	0.223	**0.659**	**0.838**	**0.898**	**0.850**	0.216	-0.349	**0.910**	0.311	**0.922**	**0.898**	0.156		
VEGF	0.089	-0.327	-0.190	-0.167	-0.238	0.524	0.012	-0.071	0.190	0.012	-0.452	0.500	-0.180	
MMP-1	0.368	0.518	**0.833**	**0.714**	**0.786**	0.357	-0.252	**0.619**	0.500	**0.659**	**0.619**	0.095	0.587	-0.214
MMP-2	-0.393	-0.103	-0.476	-0.548	-0.476	-0.548	**0.611**	**-0.762**	0.190	**-0.778**	**-0.690**	0.143	**-0.755**	0.000
MMP-3	-0.051	-0.382	**-0.667**	**-0.762**	-0.595	-0.452	0.563	**-0.810**	0.048	**-0.862**	**-0.905**	0.167	**-0.850**	0.214
MMP-7	0.469	0.027	-0.071	-0.048	-0.119	0.333	-0.132	-0.071	0.167	0.036	-0.190	**0.833**	0.060	**0.786**
MMP-8	-0.114	**0.736**	**0.643**	0.571	**0.619**	0.048	-0.144	0.286	**0.786**	0.275	0.286	0.071	0.216	-0.333
MMP-9	0.444	**0.791**	**0.881**	**0.786**	**0.881**	0.143	-0.228	**0.619**	**0.667**	**0.635**	**0.643**	0.333	**0.683**	-0.262
MMP-13	**-0.799**	-0.109	-0.238	-0.262	-0.286	**-0.643**	**0.850**	-0.429	0.048	-0.419	-0.262	-0.095	-0.395	-0.190
TIMP-1	-0.317	-0.109	0.238	0.190	0.238	0.214	0.467	0.262	0.548	0.275	-0.190	0.167	0.036	0.571
TIMP-2	-0.140	-0.218	0.286	0.238	0.333	0.405	0.204	0.452	0.405	0.419	-0.095	-0.119	0.132	0.476
TIMP-4	-0.317	**-0.791**	**-0.762**	**-0.714**	**-0.857**	0.262	0.072	**-0.619**	-0.476	-0.551	**-0.690**	-0.119	**-0.731**	0.571
MMP Act	0.063	0.436	**0.667**	0.571	**0.786**	0.048	0.072	0.595	**0.738**	0.491	0.238	-0.095	0.383	-0.167
MGAG	0.076	-0.218	0.262	0.190	0.071	**0.714**	-0.156	0.190	0.024	0.347	0.167	0.071	0.960	0.405

	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-13	TIMP-1	TIMP-2	TIMP-4	MMP Act
MMP-2	-0.452										
MMP-3	-0.476	**0.881**									
MMP-7	-0.095	-0.119	0.095								
MMP-8	0.524	0.238	-0.048	-0.333							
MMP-9	**0.905**	-0.333	-0.405	0.024	0.643						
MMP-13	-0.357	**0.714**	0.452	-0.405	0.095	-0.333					
TIMP-1	0.167	0.119	0.143	0.143	0.119	0.071	0.310				
TIMP-2	0.310	-0.167	-0.048	0.000	0.071	0.119	0.024	**0.922**			
TIMP-4	**-0.667**	0.286	0.405	0.238	-0.548	**-0.857**	0.143	0.095	0.048		
MMP Act	**0.643**	-0.048	-0.095	-0.333	**0.690**	**0.667**	0.000	0.524	**0.619**	**-0.643**	
MGAG	0.524	-0.405	-0.310	0.238	-0.119	0.190	-0.262	0.357	0.452	0.238	0.000

Table 5-6: Trends among media protein concentrations. r>0.6 or <-0.6 indicated by bold numbers.

	Median	Interquartile Range
IL-2 (pg/mL/g)	0.45	0.00-1.13
IL-4 (pg/mL/g)	0.00	0.00-2.49
IL-6 (pg/mL/g)	5344.10	2048.12-11580.47
IL-8 (pg/mL/g)	10544.50	5301.21-16806.68
IL-10 (pg/mL/g)	102.78	24.59-188.71
NO (pg/mL/g)	0.23	0.19-0.41
PGE_2 (pg/mL/g)	1142.89	220.27-4529.70
GRO-α (pg/mL/g)	1984.08	1546.40-6608.33
MCP-1 (pg/mL/g)	12207.22	7423.68-17574.58
MIP-1α (pg/mL/g)	65.52	27.29-2962.81
MIP-1β (pg/mL/g)	72.18	16.72-192.26
RANTES (pg/mL/g)	200.36	105.45-320.57
TNF-α (pg/mL/g)	10.31	1.25-361.51
VEGF (pg/mL/g)	3979.71	1265.17-6121.42
MMP-1 (ng/mL/g)	231.28	18.59-272.15
MMP-2 (ng/mL/g)	80.38	60.19-376.02
MMP-3 (ng/mL/g)	796.57	81.29-3091.91
MMP-7 (pg/mL/g)	2867.01	1562.59-3295.10
MMP-8 (pg/mL/g)	1360.34	495.27-1830.17
MMP-9 (pg/mL/g)	637.08	303.42-1000.53
MMP-13 (ng/mL/g)	28.96	9.99-154.67
TIMP-1 (ng/mL/g)	172.30	115.95-298.84
TIMP-2 (ng/mL/g)	41.79	19.37-55.43
TIMP-4 (pg/mL/g)	821.45	18.59-272.15
MMP Act (ng/mL/g)	476.31	192.01-605.58
MGAG (mg/mL/g)	10.32	8.02-16.53

Table 5-7: Median and interquartile range of biomarker concentration standardized to tissue wet weight.

CHAPTER 6:

CANINE HIP DYSPLASIA: CELLULAR MECHANOBIOLOGICAL RESPONSES OF INTRA-ARTICULAR TISSUES

Research Objective and Hypothesis:

Developmental dysplasia of the hip (DDH) results in abnormal biomechanical loads, torques, and translations on the articular and peri-articular tissues.[1,2] Characterizing cell and tissue mechanobiology to determine the related effects on hip joint health and function is critical for elucidating clinically relevant biomarkers and targets for prevention and treatment of DDH. Importantly, canine hip dysplasia closely mimics DDH in humans with respect to biomechanical perturbations and the associated pathology, symptomology, diagnostic findings, and treatment options.[3,4] Therefore, canine tissues can be effectively used to investigate the mechanobiologic effects of hip dysplasia on the clinically relevant tissues toward the goal of delineating valid biomarkers and therapeutic targets. The tissues analyzed in this study were the acetabular labrum, ligamentum teres of the femoral head, and the synovium. These tissues were selected as they have been described to provide stability to the hip joint and previous research has shown that these tissues can be overloaded in the setting of DDH.[5–10] The objective of this study was to investigate the effects of mechanical loading on the biochemical responses of cells from these tissues that were recovered from dysplastic and healthy hips. It was hypothesized that cellular metabolic responses relevant to

mechanobiologic pathways of DDH would be significantly different based on tissue type and applied tensile load.

Material and Methods:

Tissue Collection and Culture:

With ACUC approval (#9961 and 11680) tissue samples were recovered from purpose bred research hounds (n=8, 8 female) that were euthanized for reasons unrelated to this study. At time of euthanasia, radiographs in a straight, extended-leg ventrodorsal view of the pelvis were taken and graded for presence of hip dysplasia by a board-certified veterinary radiologist based on the Orthopedic Foundation for Animals (OFA) scoring system.[11] The intra-articular soft tissues shown to provide hip stability, acetabular labrum, ligamentum teres (LT), and the synovium, were collected from 8 left hips and 7 right hips. There were 8 healthy hips and 7 dysplastic hips based on the OFA scoring. Tissues were enzymatically digested with bacterial collagenase Type I for 18 hours at 5% CO2, 37°C, and 95% humidity. Cell digests were centrifuged (1500 rpm for 10 minutes) to pellet the cells to allow for the removal of the collagenase media. Cells were resuspended in 3 mL of fresh DMEM media supplemented with 1mM sodium pyruvate, 2mM L-glutamine, 0.5mg/mL ascorbic acid, 1 x MEM N-E Amino Acid solution, 1% insulin transferrin selenium (ITS premix: BD Biosciences, Bedford, MA, USA), and 1x penicillin-streptomycin-amphotericin B (all components from Invitrogen Co., Carlsbad, CA, USA unless otherwise specified)) and 10% fetal bovine serum (FBS). The fresh cell suspension was plated on a T-25 flask (Thermo Fisher

Scientific Inc., Waltham, MA, USA). Cell cultures had a media change every 3 days until cells reach >80% confluency. Once confluent, the cells were released from the flask with TrypLe (Thermo Fisher Scientific Inc., Waltham, MA, USA) and counted using the Trypan Blue (Thermo Fisher Scientific Inc., Waltham, MA, USA) exclusion assay.[12] Cells were aliquoted at $1x10^6$ cells per 1 mL aliquot and stored in freezing media consisting of DMEM supplemented with 1mM sodium pyruvate, 2mM L-glutamine, 0.5mg/mL ascorbic acid, 1 x MEM N-E Amino Acid solution, and 1x penicillin-streptomycin-amphotericin B (all components from Invitrogen Co., Carlsbad, CA, USA unless otherwise specified)) with 20% FBS and 10% dimethyl sulfoxide (DMSO). Cells were stored in liquid nitrogen until used for loading cultures.

Mechanical Stimulation:

Cells were removed from liquid nitrogen and thawed. Cells were resuspended in DMEM media supplemented with 1mM sodium pyruvate, 2mM L-glutamine, 0.5mg/mL ascorbic acid, 1 x MEM N-E Amino Acid solution, 1% insulin transferrin selenium (Thermo Fisher Scientific Inc., Waltham, MA, USA), 1x penicillin-streptomycin-amphotericin B (all components from Invitrogen Co., Carlsbad, CA, USA unless otherwise specified)), and 10% FBS. Cells suspensions were centrifuged (1500 rpm for 10 minutes) to pellet the cells and all media was removed. The cells were resuspended in fresh 10% FBS supplemented media as described above. Live cells were counted in utilizing Trypan Blue (Thermo Fisher Scientific Inc., Waltham, MA, USA) exclusion assay.[12] Cells were placed onto

Flexcell CellSoft 6 well BioFlex plates (Flexcell International Corp., Burlington, NC, USA) treated with covalently bonded collage type I protein matrix to allow for better cell adhesion at a density of 100,000 cells per well. 10% FBS supplemented DMEM with 1mM sodium pyruvate, 2mM L-glutamine, 0.5mg/mL ascorbic acid, 1 x MEM N-E Amino Acid solution, 1% insulin transferrin selenium (Thermo Fisher Scientific Inc., Waltham, MA, USA), and 1x penicillin-streptomycin-amphotericin B (all components from Invitrogen Co., Carlsbad, CA, USA unless otherwise specified)) was placed on cells for 3 days to allow for growth to >80% confluency then media was removed and replaced with 2% FBS supplemented DMEM with 1mM sodium pyruvate, 2mM L-glutamine, 0.5mg/mL ascorbic acid, 1 x MEM N-E Amino Acid solution, 1% insulin transferrin selenium (Thermo Fisher Scientific Inc., Waltham, MA, USA), and 1x penicillin-streptomycin-amphotericin B (all components from Invitrogen Co., Carlsbad, CA, USA unless otherwise specified)) for 24 hours.

After 24 hours fresh media was replaced, and cells underwent their given loading protocol. Because these tissues undergo loading in more than one plane during hip movement, an equibiaxial tension load was applied to the cells during culture using the Flexcell FX-4000 Tension System (Flexcell International Corp., Burlington, NC, USA).[13–15] A tension load of 0%, 4%, or 8% was applied to the cells to model unloading, physiological load (health hip), and supraphysiological (DDH hip) loads that can occur in the healthy and DDH hip.[16–18] The tension load was applied cyclically to the cells for 48 hours using a half sine wave at 1 Hz for 2

seconds followed by an 8 second rest period for 16 hours, and then unloaded for 8 hours.

The frequency and load protocol was selected to model the average walking speed of a human with sufficient rest to prevent cells from detaching from the wells.[19] Our goal was to cyclically load the tissues to the approximate steps they would see in a day, spread out evenly across the time they would see activity, allowing for sufficient cell resting to model native activity. With this goal, we aimed for around 10,000 steps, a day as this is the commonly accepted threshold between somewhat active and regularly active, while 16 hours of total activity matches the accepted 16 hours of wakefulness a human has in a 24-hour circadian rhythm.[20,21] With this, the protocol stated above the cells were cyclically stretched 11,520 times over the 16 hour period of activity. At the end of culture, the media were collected and stored at -20ºC until used for protein analysis.

Resazurin Assay:

The resazurin assay was completed using the Resazurin sodium salt (Thermo Fisher Scientific Inc., Waltham, MA, USA). In the 6 well plate containing the 5 mL of media and cell 1 mL of working concentration of resazurin was added to the well. The plates were placed in the incubator at 37°C for 1 hour. After the hour of incubation 100uL of media sample was collected and red for resorufin produced during the incubation to allow for cellular activity to be quatified.[22]

Protein Analysis:

Culture media were tested for interleukin (IL)-6, IL-8, IL-10, monocyte chemoattractant protein (MCP)-1 using the Milliplex Canine Cytokine/Chemokine Magnetic Bead Multiplex Assay (MilliporeSigma, Burlington, MA, USA), matrix metalloprotease (MMP)-1, MMP-2, MMP-3, (R&D Systems, Minneapolis, MN, USA) using Magnetic Luminex Perfomance Assay MMP Base Kit. The osteocalcin (OC) using the Canine Osteocalcin ELISA Kit assay (MyBioSource, San Diego, CA). The concentration of prostaglandin E2 (PGE_2) was measured using the Prostaglandin E_2 Express ELISA Kit (Cayman Chemical Co., Ann Arbor, MI). The concentration of glycosaminoglycans (GAG) was determined using the dimethylmethylene blue (DMMB) assay, and nitric oxide (NO) using the 2,3-Diaminomaphthalene (DAN) assay, as previously described.[23,24]

Statistical Analysis:

Data were checked to determine normality and all data were determined to be non-normal. A Kruskal-Wallis test, followed by a Dunn post-hoc test with Bonferroni correction, were employed for differences between tissue types, strain, the combination of tissue type with strain, and the combination of hip status with tissue type and considering the difference strains with significance set at $p<0.05$. A Mann-Whitney U test with Bonferroni correction was employed when comparing the hip status within each tissue type regardless of the strain with significance set at $p<0.05$. Analyses were performed in R version 4.1.2 (R Core Team, 2021) and figures were produced using the ggplot2 package (v3.3.5; Wickham H, 2016).

Results:

Statistically significant differences are presented.

Load-dependent Differences within Tissue Types:

When comparing how load affected the production in each specific tissue type, the p-values and the concentrations were reported. **(Table 6-1 & 6-3, Figure 6-1)**. For labrum tissues, nitric oxide released to the media was significantly higher under 8% tensile loading compared to 0% loading ($p=0.03$). For LT tissues, the production of IL-6 was significantly increased when loaded at 8% strain compared to 0% ($p=0.008$) and 4% ($p=0.01$) strain groups. Production of PGE_2 was significantly increased under 8% load compared to 0% ($p=0.03$) and 4% ($p=0.07$) strain groups. GAG released to the media was significantly higher at 8% load compared to 0% strain group ($p=0.01$). Nitric oxide released to the media was significantly higher at 8% load compared to 0% ($p=0.002$) and 4% ($p=0.0002$) load.

Differences between Tissue Types:

There were no significant differences in biomarker concentrations in those with dysplasia to those in healthy hips, within each tissue type regardless of the strain applied. **(Table 6-2 & 6-3)**

When the data from the three tissue types were compared between strains **(Table 6-1 & 6-3, Figure 6-2)**, it was observed that there no significant differences in media biomarker concentrations between tissue types at 0% strain. Further, when the cells were cultured under 4% strain the only significant difference observed was the production of OC was significantly ($p=0.015$) higher by the labral

cells compared to the LT cells. Therefore, there does not appear to be a significant difference in the inflammatory and degradative metabolic responses to strain by the cells from these intra-articular tissues of the hip when strain deprived (0%) and at physiological (4%) strain levels.

However, at the supraphysiological 8% strain level, designed to model strains encountered during DDH, the LT appeared to have a higher pro-inflammatory response compared to the synovium. The LT cells produced significantly higher levels of IL-6 ($p=0.01$), PGE_2 ($p=0.008$), and NO ($p=0.009$) compared to synovial cells cultured at 8% strain. Further, LT cells produced significantly ($p=0.03$) higher levels of PGE2 compared to labral cells when cultured at 8% strain. Therefore, the LT may have a higher inflammatory response to the high strain levels experience during DDH compared to the labrum and the synovium. Additionally, LT cells produced significantly ($p=0.015$) higher levels of GAG compared to synovial cells cultured at 8% strain, indicating a potential increase in ECM matrix production and tissue remodeling response by the LT compared to the synovium at this higher strain level.

Hip Status Differences with Strain and in Tissue Type:

The comparison of hip status within each strain and tissue type's p-value and concentrations were reported. **(Table 6-4, 6-5, 6-6, & Figure 6-3)**

For **labrum**, cells from dysplastic hips produced significantly higher levels of MCP-1 ($p=0.02$) at 8% load compared to healthy-hip cells. For **LT**, cells from

dysplastic hips produced significantly higher levels of MCP-1 ($p=0.04$) at 8% load compared to healthy-hip cells.

Discussion:

This study investigated the effects of differential tensile loading responses of cells from intra-articular tissues in healthy and dysplastic hips. The results from this study provided evidence in support of the hypothesis that cellular metabolic responses relevant to mechanobiologic pathways of dysplasia would be significantly different based on tissue type and applied tensile load. Specifically, the results provided evidence that the ligamentum teres and acetabular labrum were responders to increased load based on increased expression of inflammatory and bone metabolism related biomarkers, while there was no evidence that the synovium increased relevant biomarker production in association with increased tensile loading in this study. When ligamentum teres and labrum from dysplastic hips were placed under physiologic load their biomarker production was not significantly different from the same tissues recovered from healthy hips.

In hip dysplasia, intra-articular tissues including acetabular labrum, ligamentum teres, and synovium are placed under abnormal biomechanical loads, torques, and strains due to the shallowness of the acetabulum and instability of the hip.[6–10] In the present study, ligamentum teres cells under supraphysiologic tensile load produced higher levels of IL-6, IL-8, nitric oxide and GAG and labrum cells produced higher levels of nitric oxide when compared to synovial cells under the same loading conditions. Supraphysiologic tensile loading was also associated

with increased production of PGE_2 by ligamentum teres cells compared to synovium and labrum cells. IL-6 and IL-8 are cytokines which have been implicated in inflammatory processes in joints, and IL-8 has been associated with chondrocyte hypertrophy while IL-6 has been associated with production of metalloproteinases in a mouse model.[25,26] Load-dependent increases in PGE_2 could be expected to be part of the same inflammatory pathway based on the documented relationships with IL-6, IL-8, and pain-signaling mediators in similar tissues.[27–31] The data from the present study support these linked metabolic responses from ligamentum teres cells subjected to supraphysiological tensile loading, highlighting this load-dependent inflammatory pathway as a potential mechanism related to hip instability.

In addition to load-dependent differences in cellular metabolic responses, there were differences in responses based on hip status. For dysplastic ligamentum teres cells under supraphysiologic load, there were significantly higher levels of MCP-1 compared to ligamentum teres cells from healthy hips. The upregulation of MCP-1 associated with dysplasia suggests a mechanobiologic response to ligamentum teres overstretching to recruit monocytes and signal repair processes in conjunction with early degeneration as a result of tissue damage.[32,33] Labral cells from dysplastic hips produced significantly higher levels of MCP-1 compared to labral cells from healthy hips when exposed to a supraphysiologic level of strain (8%) during culture. Interestingly, dysplastic-related differences in metabolic responses to supraphysiologic tensile loading by ligamentum teres and acetabular labrum cells were not maintained under physiologic loading. These

results indicate that soft tissue overloading can drive complex inflammatory pathways involved in hip degeneration and osteoarthritis and that restoration of physiologic loading can normalize metabolic response profiles for these tissues. These results may indicate overstretching of these tissue types may add to an inflamed environment in the joint and drive OA. Furthermore, these increases have been implicated as being downstream effects of TLR4, which suggests a mechanotransduction pathway for the biomarker changes noted.[34,35]

While this study was completed on canine tissues, it is generally accepted that canine hip dysplasia (CHD) and developmental dysplasia of the hip (DDH) in humans involve similar pathomechanisms and progression to secondary hip osteoarthritis, making CHD an ideal translational model for DDH.[3,4] However, this study was designed as a monolayer cell culture experiment such that the effects of the extracellular matrix were not considered. Further, each tissue studied was comprised of multiple cell types, while monolayer culture inherently selects for fibroblasts and alters phenotype. However, cell culture studies allow for clinically relevant loading and control of variables such that mechanistic responses and associated biomarkers can be characterized as a foundational step towards *in vivo* and clinical studies.[36] There was a single 48-hour time point analyzed such that the results can only be considered to reflect acute responses to mechanostimulation. Finally, only biaxial tensile loads were applied to the cells, which do not fully model the complex forces placed on the tissues in dysplastic hips, but allow for application of standardized clinically relevant loads of different magnitudes to be applied such that fair comparisons can be made.[13]

Taken together, the results of this experiment suggest that ligamentum teres and acetabular labrum cells respond to supraphysiologic tensile loading by acute release of inflammation-related biomarkers. These results provide potential therapeutic targets for mitigating the mechanobiological responses associated with the development and progression of hip dysplasia. Interestingly, physiologic loading of ligamentum teres and acetabular labrum cells from dysplastic hips was associated with biomarker profiles that were not significantly different from those from the corresponding cells from healthy hips. These results highlight the importance of addressing hip joint overloading as part of a comprehensive strategy to prevent the progression of dysplasia to secondary hip OA. These experiments should be repeated using human cells and then on a translational pathway of preclinical and clinical studies to validate these results for application to DDH.

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Tissues: Strain Differences	Rezasurin	MMP-2	MMP-1	MMP-3	IL-6	IL-8	MCP-1	PGE_2	Media GAG	Nitric Oxide	OC
Ligamentum teres	0.64	0.14	0.053	0.06	0.003 *	0.037 *	0.19	0.006 *	0.011 *	0.0001 *	0.09
0% vs 4%					1	1		1	1	1	
0% vs 8%					0.008 *	0.09		0.03 *	0.0097 *	0.002 *	
4% vs 8 %					0.01 *	0.06		0.007 *	0.099	0.0002 *	
Labrum	0.47	0.56	0.28	0.61	0.84	0.38	0.77	0.74	0.4	0.004 *	0.25
0% vs 4%										0.8	
0% vs 8%										0.003 *	
4% vs 8 %										0.11	
Synovium	0.15	0.42	0.29	0.18	0.12	0.31	0.1	0.17	0.095	0.86	0.52

Table 6-1: Significant difference between strains within tissue type with p-values reported. (*=statistically significant difference ($p<0.05$) based on Kruskal-Wallis following a Dunn post-hoc analysis and Bonferroni correction)

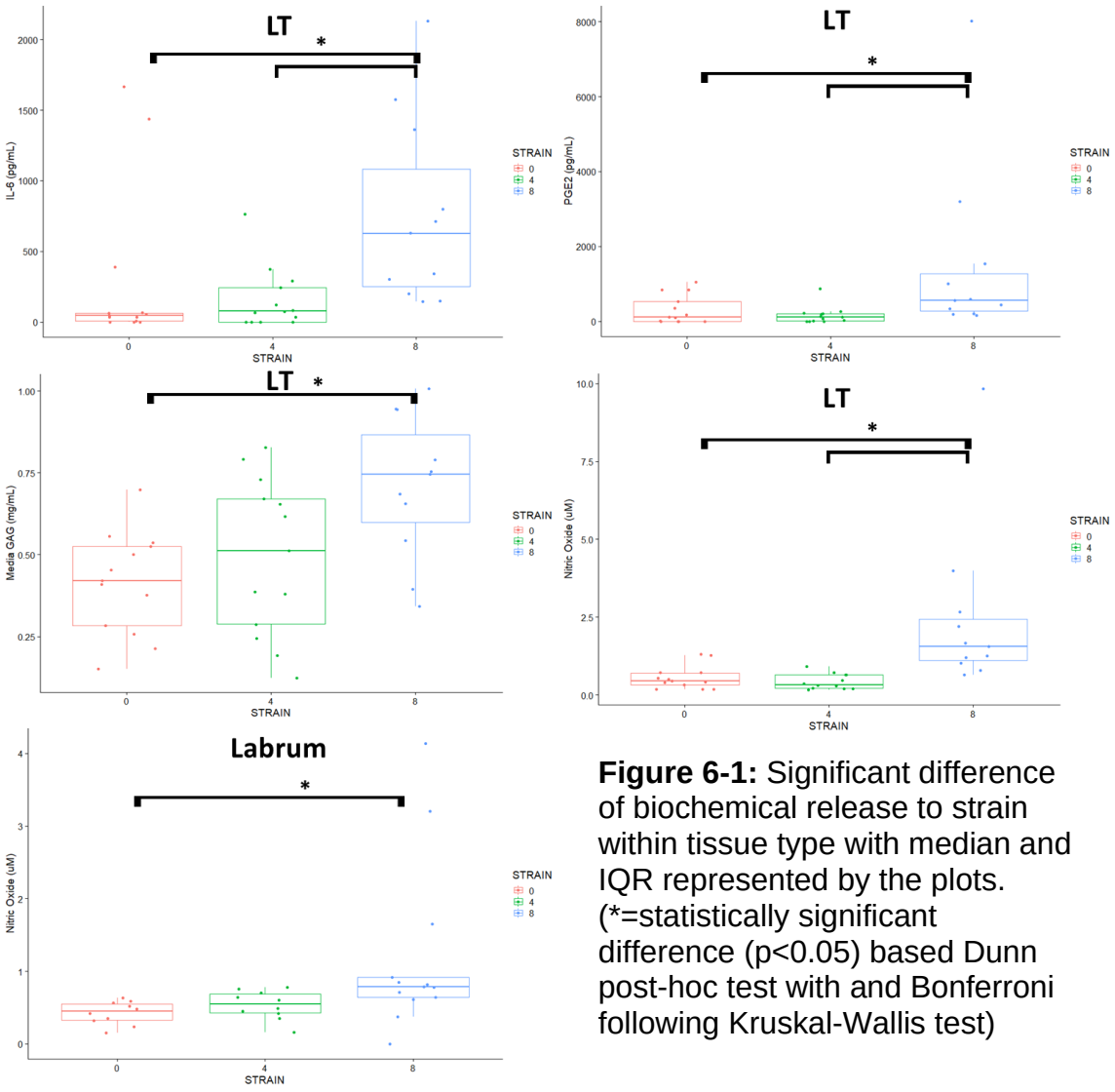

Figure 6-1: Significant difference of biochemical release to strain within tissue type with median and IQR represented by the plots. (*=statistically significant difference ($p<0.05$) based Dunn post-hoc test with and Bonferroni following Kruskal-Wallis test)

	Rezasurin	MMP-2	MMP-1	MMP-3	IL-6	IL-8	MCP-1	PGE_2	Media GAG	Nitric Oxide	OC
DDH vs. Healthy											
Ligamentum teres	0.71	0.46	0.59	0.95	0.23	0.39	0.76	0.93	0.95	0.43	0.68
Labrum	0.9	0.86	0.72	0.64	0.4	0.9	0.47	0.43	0.38	0.61	0.71
Synovium	0.92	0.45	0.26	0.96	0.37	0.26	0.77	0.69	0.48	0.57	0.84
Strain: Tissue Differences											
0	0.86	0.89	0.73	0.38	0.47	0.95	0.8	0.48	0.67	0.75	0.83
4	0.76	0.27	0.06	0.08	0.13	0.46	0.13	0.26	0.87	0.41	0.02 *
Labrum vs. Ligamentum Teres											0.015 *
Labrum vs. Synvoium											0.83
Ligamentum teres vs Synovium											0.24
8	0.66	0.83	0.5	0.16	0.013 *	0.06	0.14	0.006 *	0.01 *	0.009 *	0.54
Labrum vs. Ligamentum Teres					0.33			0.03 *	0.057	0.08	
Labrum vs. Synvoium					0.46			1	1	1	
Ligamentum teres vs Synovium					0.01 *			0.008 *	0.015 *	0.009 *	

Table 6-2: Significant difference between dysplastic and healthy hips within tissue types (based on Mann-Whitney U and Bonferroni correction) and tissue type differences within strains with p-values reported. (*=statistically significant difference ($p<0.05$) based on a Dunn post-hoc test with Bonferroni correction following a Kruskal-Wallis test)

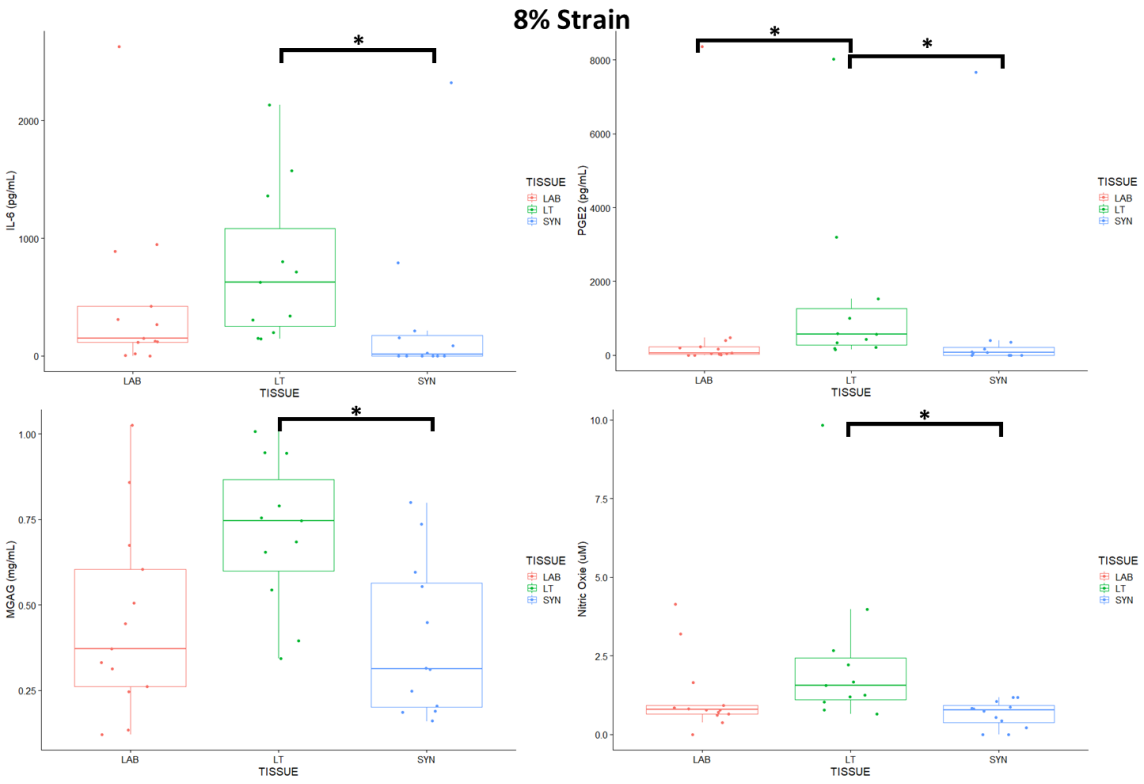

Figure 6-2: Significant difference of biochemical release of tissue types at 8% strain with median and IQR represented by the plots. (*=statistically significant difference ($p<0.05$) based on Kruskal-Wallis following a Dunn post-hoc analysis and Bonferroni correction)

		Rezasurin	MMP-2	MMP-1	MMP-3	MMP-9	IL-6	IL-8	MCP-1	PGE_2	Media GAG	Nitric Oxidie	OC
	Dysplastic	196201 (86164-270294)	42203 (35943-48420)	42.24 (0-161.51)	15.05 (0-99.11)	0 (0-0)	121.98 (0-686.54)	11204 (658.6-22429)	15435 (2641.5-34340.5)	159.56 (0-599.24)	0.48 (0.28-0.64)	0.62 (0.35-0.86)	0.51 (0-1.00)
	Healthy	147379 (53336-446729)	43367 (38183-53321)	37.46 (0-106.76)	14.75 (0-74.20)	0 (0-0)	153.79 (36.1-651.14)	12321 (4297-18035)	15447 (6496.5-23713.5)	177.76 (50.42-517.87)	0.45 (0.32-0.61)	0.56 (0.32-0.80)	0.51 (0-1.38)
0%	Labrum	241483 (76195-647466)	40906 (37647.25-47316)	56.05 (13.96-92.12)	0 (0-48.52)	0 (0-2.83)	103.47 (0-465.53)	11223 (420.14-15197.75)	13823.5 (2335.5-18282.5)	116.36 (54.20-1027.79)	0.431 (0.348-0.484)	0.45 (0.33-0.55)	0.83 (0.18-1.88)
	Ligamentum Teres	193920 (57108-432245)	43460 (35233-48548)	124.23 (0-198.01)	0 (0-67.96)	0 (0-0)	47.27 (5.95-63.55)	3935 (692.41-20654)	18083 (1953-26856)	122.93 (0-536.84)	0.421 (0.283-0.525)	0.448 (0.329-0.710)	0.61 (0.329-1.042)
	Synovium	266899 (34502-442946)	41709 (39380-43274)	87.25 (12.8-167.29)	53.39 (0-75.38)	0 (0-0)	100.11 (36.1-698.74)	7647 (4158-14041)	15410 (5492-29827)	306.07 (63.25-1664.44)	0.35 (0.29-0.45)	0.534 (0.312-0.804)	0.828 (0.175-1.462)
4%	Labrum	310952 (103539-697525)	44801 (36923-54603)	49.18 (29.65-121.42)	17.63 (0-59.83)	0 (0-8.98)	192.82 (0-997.75)	11223 (1995-17186)	22575 (5025-34728)	188.63 (0-639.96)	0.524 (0.382-0.663)	0.549 (0.427-0.686)	0.865 (0.631-1.747)
	Ligamentum Teres	231613 (85025-291993)	39045 (36477-40468)	14.49 (0-44.75)	0 (0-30.7)	0 (0-8.0)	75.93 (0-243.03)	6521 (3177-17710)	122272 (6237-19755)	109.57 (17.8-204.57)	0.512 (0.288-0.671)	0.334 (0.215-0.643)	0.221 (0-0.549)
	Synovium	257239 (102746-501864)	43246 (40454-47157)	104.21 (57.62-164.1)	58.97 (30.1-78.43)	0 (0-0)	470.31 (124.51-1086)	14527 (6555-19699)	21379 (13411-32603)	319.54 (85.442-612.12)	0.594 (0.333-0.648)	0.504 (0.378-0.648)	0.788 (0.067-1.163)
8%	Labrum	140092 (96125-200856)	45355 (38700-52754)	0 (0-86.84)	0 (0-100.75)	0 (0-0)	147.4 (115.23-421.77)	20209 (8223-22565)	15600 (6316-21546)	58.97 (35.78-231.19)	0.371 (0.261-0.604)	0.788 (0.645-0.918)	0.452 (0-1.849)
	Ligamentum Teres	126808 (96125-200856)	49745 (39972-57977)	21.91 (6.8-90.53)	82.52 (18.56-114.53)	0 (0-0)	627.14 (252.17-1081.31)	19218 (15204-25509)	18055 (13449-28046)	566.83 (274.42-1269.77)	0.746 (0.599-0.867)	1.561 (1.112-2.437)	0 (0-0.604)
	Synovium	90134 (59735-200318)	48556 (37875-65815)	0 (0-25.015)	0 (0-16.49)	0 (0-0)	11.88 (0-171.37)	5003 (629.13-15377.75)	3777 (1848-20805)	67.97 (0-218.36)	0.313 (0.201-0.565)	0.773 (0.378-0.916)	0.171 (0-0.782)

Table 6-3: Median and interquartile range of biomarkers between hip status and between tissue type, at each individual strain.

Tissue type & strain: Dysplastic vs. Healthy	Rezasurin	MMP-2	MMP-1	MMP-3	IL-6	IL-8	MCP-1	PGE_2	Media GAG	Nitric Oxide	OC
Ligamentum Teres											
0%	0.88	0.19	0.6	1	0.21	0.12	0.19	0.88	0.14	0.31	0.94
4%	0.56	0.31	0.25	0.24	0.21	0.19	0.19	0.24	0.38	0.57	0.22
8%	0.72	0.14	0.14	0.36	0.72	0.07	0.04*	0.14	0.72	0.58	0.43
Labrum											
0%	0.52	0.75	0.42	0.1	0.17	0.08	0.2	0.75	0.13	1	0.34
4%	0.87	0.42	0.38	0.31	0.29	0.52	0.63	0.22	0.26	1	0.15
8%	0.67	0.67	0.09	0.12	0.09	0.007	0.02*	0.57	0.25	0.15	0.22
Synovium											
0%	0.89	0.06	0.67	0.77	0.053	0.06	0.48	0.32	0.52	0.92	0.11
4%	0.57	0.09	0.053	0.77	0.72	0.32	0.57	0.94	0.78	0.94	0.2
8%	0.44	0.29	0.93	0.34	0.54	0.81	0.57	0.51	0.17	0.29	0.93

Table 6-4: Significant difference between dysplastic and healthy hips for each tissue type and strain within tissue types. (*=statistically significant difference ($p<0.05$) based on Dunn post-hoc tests with Bonferroni correction following Kruskal-Wallis)

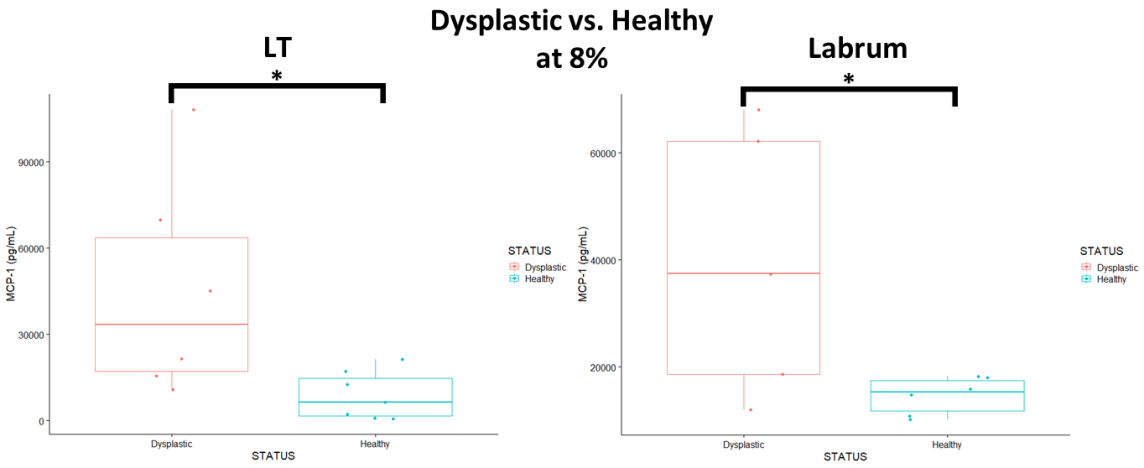

Figure 6-3: Significant differences of biochemical release to hip status for the labrum and LT tissues at 8% strain with median and IQR represented by the plots. (*=statistically significant difference ($p<0.05$) based on Dunn post-hoc test with Bonferroni correction following Kruskal-Wallis test)

			Rezasurin	MMP-2	MMP-1	MMP-3	MMP-9	IL-6	IL-8	MCP-1	PGE_2	Media GAG	Nitric Oxidie	OC
Labrum	0%	Dysplastic	237972 (85303-504618)	40906 (37054-45079)	33.36 (4.65-78.37)	0 (0-35.66)	0 (0-0)	16.88 (0-80.38)	1457.85 (123.08-8439)	3223 (194-11144)	116.36 (28.12-687.57)	0.477 (0.422-0.494)	0.451 (0.366-0.542)	0.306 (0.01-1.548)
		Healthy	241483 (38232-52198)	40262 (36023-44903)	38.57) (0-71.8)	0 (0-8.49)	172.01 (0-534.51)	12226 (393-15581)	14734 (13910-18958)	173 (80.36-1092.63)	55.23 (12.49-110.26)	0.399 (0.299-0.547)	0.609 (0.419-1.788)	0 (0-1.274)
	4%	Dysplastic	310952. (135348-574273)	45796. (44628-57566)	36.29 (23.24-53.90)	0 (0-28.54)	0 (0-0)	8.22. (0-476.71)	3645 (958.91-17388)	21832 (3585-47535)	44.96 (0-340.77)	0.485 (0.353-0.542)	0.56 (0.365-0.741)	0.707 (0.617-0.903)
		Healthy	288458 (52701-826391)	39179 (34874-49388)	81.26 (46.37-187.73)	37.59 (8.81-99.65)	3.49 (0-12.98)	585.03 (181.2-1085.25)	12365.5 (11114.75-15111)	22575 (9637-30034)	368.62 (144.79-997.99)	0.638 (0.453-0.751)	0.549 (0.48-0.616)	1.491 (0.944-2.497)
	8%	Dysplastic	1292593 (119269-200263)	44729 (38457-48429)	74.64 (8.48-167.84)	92.25 (18.51-149.88)	0 (0-0)	343.35 (160.25-815.93)	22655 (22361-24604)	33401 (17087-63626)	128.92 (41.56-349.65)	0.439 (0.342-0.632)	0.883 (0.798-1.465)	1.167 (0.46-1.901)
		Healthy	109766 (69002-210013)	45355 (39524-59189)	0. (0-8.96)	0 (0-38.03)	0 (0-0)	115.23 (8.22-230.08)	8223 (2513-14449)	6316 (1594-14825)	45.37 (21.97-201.33)	0.313 (0.191-0.525)	0.707 (0.493-0.803)	0.032 (0-0.382)
Ligamentum Teres	0%	Dysplastic	193919 (85199-288636)	57070 (41071-57353)	0 (0-222.8)	0 (0-92.11)	0 (0-0)	36.1 (5.95-47.27)	2791 (503.1-3709)	15270 (1038-18083)	14.25 (0-838.33)	0.283 (0.213-0.453)	0.512 (0.448-1.277)	0.533 (0.337-0.873)
		Healthy	257655 (38652-452309)	40819 (35191-44869)	127.93 (20.34-183.64)	28.99 (0-65.96)	0 (0-0)	62.73 (27.08-653.29)	16851 (3124-24056)	25176 (7551-28094)	152.67 (80.81-404.26)	0.461 (0.401-0.542)	0.402 (0.294-0.583)	0.693 (0.312-1.453)
	4%	Dysplastic	107202 (85025-244750)	38254 (35881-39408)	0 (0-14.49)	0 (0-0)	0 (0-13.73)	0 (0-120.29)	5054 (116.32-5575)	6237 (890.56-9813)	17.8 (0-109.57)	0.729 (0.288-0.792)	0.473 (0.305-0.643)	0 (0-0.018)
		Healthy	253074 (83118-617664)	39364 (37271-44749)	36.82 (0-108.56)	14.75 (0-52.41)	0 (0-0)	78.01 (58.37-310.74)	11753 (5685-19116)	14397 (10963-20365)	160.18 (64.20-209.85)	0.449 (0.346-0.625)	0.296 (0.21-0.503)	0.423 (0.166-0.607)
	8%	Dysplastic	145486 (96771-174254)	34153 (34029-47474)	74.3 (36.72-158.91)	102.13 (67.29-126.93)	0 (0-0)	627.14 (340.53-1363)	28082 (22935-46123)	37475 (18616-62117)	1535 (436.72-3196.18)	0.746 (0.655-0.754)	1.561 (1.257-1.668)	0.321 (0-0.731)
		Healthy	122961 (102788-132342)	54998 (50639-58628)	14.595 (3.4-20.33)	59.82 (9.278-83.54)	0 (0-0)	507.95 (226.34-777.72)	17462 (14361-18922)	15334 (11851-17510)	453.12 (226.45-578.88)	0.738 (0.579-0.906)	1.93 (1.07-3.66)	0 (0-0.357)

Table 6-5: Median and interquartile range of biomarkers for the labrum and ligamentum teres tissue between dysplastic and healthy hips at each strain level, within tissue type.

			Rezasurin	MMP-2	MMP-1	MMP-3	MMP-9	IL-6	IL-8	MCP-1	PGE_2	Media GAG	Nitric Oxidie	OC
Synovium	0%	Dysplastic	266899 (121892-301035)	40369 (36875-42203)	87.25 (71.67-166.08)	53.39 (16.11-85.71)	0 (0-0)	698.74 (148.58-1218.02)	13300 (9192-19497)	21379 (8070-34657)	507.67 (184.66-4328.53)	0.419 (0.308-0.507)	0.788 (0.309-0.809)	0.192 (0.088-0.641)
		Healthy	260761 (45520-445783)	43424 (41685-53251)	56.88 (3.2-150.71	35.33 (0-74.20)	0 (0-5.235)	50.65 (20.29-79.48)	4436 (2426-5195)	14829 (7681-15465)	186.23 (59.72-331.51)	0.344 (0.303-0.366)	0.32 (0.317-0.748)	1.328 (0.86-1.811)
	4%	Dysplastic	238802 (154660-266764)	46942 (44300-50858)	164.1 (116.37-200.5)	58.97 (39.47-98.65)	0 (0-0)	674.33 (159.5-1085.78)	16653 (10541-21795)	27552 (16142-57902)	319.54 (147.58-603.54)	0.594 (0.445-0.639)	0.571 (0.372-0.719)	0.953 (0.585-1.26)
		Healthy	4133352 (99304-502824)	40515 (32537-40718)	69.8 (32.4-91.78)	57.66 (14.00-61.54)	0 (0-0)	313.54 (132.58-932.08)	13126 (7223-16856)	20577 (15002-27200)	266.58 (59.29-1226.03)	0.566 (0.382-0.767)	0.448 (0.441-0.56)	0.394 (0-0.794)
	8%	Dysplastic	64405 (56389-174843)	38742 (35422-59470)	0 (0-18.115)	0 (0-0)	0 (0-0)	0 (0-90.69)	4100 (444-11663)	2815 (1833.26-12202)	14.38 (0-212.25)	0.247 (0.188-0.433)	0.536 (0.109-0.854)	0.045 (0-0.861)
		Healthy	114533 (65734-198793)	57411 (51445-63329)	0 (0-37.84)	0 (0-65.95)	0 (0-0)	86.73 (0-212.6)	8797 (1969-14697)	9012 (1937-24220)	96.32 (65.56-173.12)	0.448 (0.315-0.596)	0.806 (0.74-1.049)	0.296 (0-0.329)

Table 6-6: Median and interquartile range of biomarkers for synovium tissue between dysplastic and healthy hips at all strain levels.

CHAPTER 7:

EVALUATION OF SERUM AND URINE BIOMARKERS FOR SECONDARY OSTEOARTHRITIS ASSOCIATED WITH DEVELOPMENTAL DYSPLASIA OF THE HIP

Research Objective and Hypothesis:

Developmental dysplasia of the hip (DDH) is a significant cause of early-onset hip osteoarthritis (OA).[1–3] However, the pathomechanisms involved in the progression of DDH to hip OA have not been fully elucidated. Based on ethical, financial, and access limitations for invasive assessments in conjunction with the limited capabilities for non-invasive modalities, analysis of biomarkers in bodily fluids may be the most optimal method available to evaluate potential pathomechanisms of DDH's progression to hip OA.[4,5]

The systemic fluids, serum and urine, are targeted for development of clinically applicable biomarkers for DDH, and are of particular interest as these fluids are easy to obtain in a minimally- or non-invasive manner as standard of care.[6–8] Recent studies have reported important correlations for serum and urine biomarkers with the progression of osteoarthritis base on radiographic severity.[4,5,9–12]

The present study was designed to apply this approach to DDH by testing the hypotheses: 1) patients with dysplastic hips without radiographic degeneration (DDH cohort) will have significant differences in concentrations of serum and/or urine protein biomarkers that mechanistically differentiate them from patients with

DDH with radiographic degeneration (DDH & OA cohort); 2) patients with DDH with radiographic degeneration (DDH & OA cohort) will have significant differences in concentrations of serum and/or urine protein biomarkers from their healthy-hip controls (controls for DDH & OA cohort); and 3) young patients with healthy hips (controls for DDH cohort) will not have significant differences in concentrations of serum and/or urine protein biomarkers compared to older patients with healthy hips (controls for DDH & OA cohort).

Materials and Methods:

Patient Population:

With IRB approval (IRB #2012192) and informed patient consent, blood and urine were collected from four cohorts of patients:

- DDH cohort (n=32, 4 male and 28 female): Young (13-34 years old) patients with physician confirmed DDH prior to clinical or radiographic signs of secondary hip OA.
- Controls for DDH cohort (n=35, 11 male and 24 female): Healthy-hip controls aged 13-34 years old with no clinical or radiographic signs of DDH.
- DDH & OA cohort (n=7, 1 male and 6 female): Middle-aged adult (35-54 years old) patients with confirmed hip OA, secondary to DDH.
- Controls for DDH & OA cohort (n=12, 2 male and 10 female): Healthy-hip controls 35-54 years old with no clinical or radiographic signs of DDH or hip OA.

Exclusion criteria included incompetent adults, prisoners, patients with cancer or who have received cancer treatment within the past 6 months of the clinic visit, patients who had any surgery within the last 6 months of the clinic visit, history of previous hip surgeries, recent joint trauma to any joint, metabolic disorders, rheumatoid arthritis, corticosteroid injections within the last 6 months of the clinic visit, currently taking oral corticosteroids, serious organ diseases or failures, are pregnant or lactating, or syndromic diseases (e.g. Cystic fibrosis or multiple sclerosis). For those identified in the DDH cohorts, DDH was determined based on physical examination by a board-certified orthopaedic surgeon in conjunction with diagnostic imaging assessments of the hips, including anteroposterior, Dunn, false-profile, and lateral radiographic views at the previous or current clinic visit. For those identified in the DDH cohorts, computed tomography (CT) was utilized to determine the presence of acetabular version and/or femoral torsion at the previous or current clinic visit. Measurements of left center-edge angle, anterior center-edge angle, Tönnis angle, acetabular extrusion index, alpha angle, head sphericity, acetabular version, and femoral torsion were also utilized for diagnosis **(Figure 2-5, 2-7, 2-8, 2-9)**. Hips were categorized by definitive diagnosis by clinical exam for the DDH cohorts while the control cohorts were volunteers with self-reported healthy hips.

Sample Collection and Storage

Blood and urine samples were collected from participants in clinic or prior to surgical intervention. Whole blood (2-6 ml) was collected by aseptic peripheral

venipuncture in a Vacutainer Serum Tube (Becton, Dickinson and Company, Franklin Lakes, NJ) and urine (>4 ml) was collected by voluntary micturition. After sample collection, the fluids were immediately transported to an on-site laboratory for processing. Whole blood samples were centrifuged (1200xg, 10 minutes), followed by serum collection. Serum and urine samples were aliquoted and stored at -80°C for subsequent analyses.

Protein Analysis

Cross linked C-telopeptide of type I collagen (CTX-I) and type II collagen (CTX-II), procollagen I C-terminal propeptide (PICP) and procollagen II (PIICP) using commercially available enzyme-linked immunosorbent assays (ELISA) assays (ABclonal; Woburn, MA). The concentration of hyaluronan (HA), a disintegrin-like and metalloproteinase domain with thrombospondin motifs 4 (ADAMTS4) and ADAMTS5, receptor activator of NF-kappa B ligand (RANKL), and Cartilage Oligomeric Matrix Protein (COMP) in the samples were assessed using DuoSet ELISA assays according to the manufacturer's protocol (R&D Systems; Minneapolis, MN). Human Multiplex Luminex immunoassays were used to analyze matrix metalloproteinase (MMP)-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-13 (R&D System, Minneapolis. MN). Human bone metabolism multiplex Luminex immunoassays were used to analyze dickkopf-related protein 1 (DKK-1), osteoprotegerin (OPG), osteocalcin (OC), osteopontin (OPN), sclerostin (SOST), parathyroid hormone (PTH) (MilliporeSigma, Burlington, MA). Human cytokine/chemokine multiplex Luminex immunoassays were used to analyze

fibroblast growth factor (FGF)-2, Fractalkine, interferon gamma (IFN-γ), growth-regulated oncogene-alpha (GRO-α), interleukin (IL)-1beta (1β), IL-1-receptor antagonist (1RA), IL-4, IL-6, IL-10, and IL-13, monocyte chemoattractant protein (MCP)-1 and MCP-3, platelet derived growth factor (PDGF)-AA and PDGF-AABB, macrophage inflammatory protein(MIP)-1 alpha (1α) and MIP-1 beta (1β), regulated on activation, normal T expressed and secreted (RANTES), tumor necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF) (MilliporeSigma, Burlington, MA). Human TIMP multiplex Luminex immunoassay bead panel 2 were used to analyze tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, TIMP-3, and TIMP-4 (MilliporeSigma, Burlington, MA) in serum and urine. The urine creatinine concentration was measured with the creatinine colorimetric assay (Cayman Chemical Company; Ann Arbor, MI), and was used to standardize the urinary concentrations obtained for the other assays.

Statistical Analysis

Data were summarized and non-normality was determined using Shapiro-Wilk normality tests. Therefore, a Mann-Whitney U test was performed to determine significant differences in biomarker concentrations associated with progression from hip dysplasia to secondary osteoarthritis (DDH vs DDH & OA), secondary osteoarthritis to healthy-hip controls (DDH & OA vs. DDH & controls), and age-related differences within healthy control samples (controls for DDH vs controls for DDH & OA). Significance was determined by a two-sided $p < 0.05$. Analysis was performed in R version 4.1.2 (R Core Team, 2021), tables were

produced using the package ggpubr (v0.4.0; Kassambara, 2020), and figures were produced using the ggplot2 package (v3.3.5; Wickham H, 2016).

Results

Progression from DDH to Secondary OA

In the urine, the DDH & OA cohort had statistically significant increases in median concentrations of SOST (p=0.036), MMP-7 (p=0.039), and TIMP-3 (p=0.027) when compared to the DDH cohort. **(Table 7-1**, denoted by "*"**)** In the serum, the DDH & OA cohort had statistically significant decreases in median concentrations of FGF-2 (p=0.027), MCP-3 (p<0.001), IL-13 p=0.003), (p=0.001), IL-4 (p=0.002), and IL-6 (p=0.001), when compared to the DDH only cohort. **(Figure 7-1, Table 7-2**, denoted by "*"**)**

Separating secondary OA from age related changes in the hip

In the urine, the DDH & OA cohort had statistically significant increases in median concentrations of PIICP (p=0.048), FGF (p=0.013), and PDGF-AB/BB (p=0.045), when compared to DDH & OA controls. **(Table 7-1**, denoted by "#"**)** In the serum, the DDH & OA cohort had statistically significant decreases in median concentrations of OC (p=0.011), MCP-1 (p=0.003), and MCP-3 (p=0.043), when compared to the DDH & OA controls. **(Figure 7-2, Table 7-2,** denoted by "*"**)**

Age related changes in the hip

In the urine, younger patients with healthy hips had statistically significant decreases in median concentrations of MCP-1 (p=0.031) and TIMP-1 (p=0.02), when compared to middle-aged patients with healthy hips. **(Figure 7-3, Table 7-1**, denoted by "#"**)** There were no significant difference in the serum median concentrations of biomarkers.

Discussion

The results of this study provide evidence to support our hypotheses that there are significant differences in concentrations of serum and urine biomarkers when comparing among participants with non-degenerative DDH (prior to degeneration, i.e. DDH only) to participants with degenerative DDH (DDH with secondary OA) and to healthy-hip controls. We rejected our hypothesis that there would not be any significant differences in concentrations of serum and urine biomarkers between healthy-hip controls in the two different age cohorts.

Non-degenerative DDH was associated with increases in catabolic, FGF-2, and inflammation related, MCP-3, IL-1RA, IL-4, IL-6, and IL-13, and decreases in degradation related, MMP-7, anti-degradative related, TIMP-3, and bone metabolism related SOST biomarkers when compared to degenerative DDH with secondary hip OA (DDH vs DDH & OA). FGF-2 has been described as acting on FGFR involving a complex cascade following the activation by FGF-2 and *in vitro* recent research has indicated that FGF-2 induces catabolic effects on human articular cartilage.[13,14] IL-1RA, IL-4, and IL-13 are all described at anti-inflammatory in the context of osteoarthritis. IL-1RA is known as a potent blocker for the IL-1

receptor to inhibit IL-1β and has been tested as an OA drug treatment. IL-4 has been described as protecting proteoglycan from MMP degradation and IL-13 has been shown, in studies of synovium samples, to inhibit the effects of an inflammatory stimulus.[15–19] As an inflammatory cytokine, IL-6 has been shown to be increased in synovial fluid from joints afflicted with OA and causes suppressed collagen type II neo-synthesis, enhanced IL-1β-mediated proteoglycan degeneration, and induction of MMP-13.[20–22]

MMP-7 is a matrilysin that is known to break down important ECM components of cartilage, in particular proteoglycans, and has often been described as being upregulated with OA. In the present study, we observed a decrease in concentrations from those with DDH compared to those with DDH and secondary OA.[23] In contrast, TIMP-3 is an anti-degradative enzyme that blocks MMPs and ADAMTS and is often decreased with the progression of OA, as observed in the present study.[24,25] Urine and serum biomarker data suggest that non-degenerative (no OA) DDH in younger patients is characterized by catabolic and inflammatory responses of the hip to the pathological state. Inflammatory biomarkers demonstrated many changes between hip states, potentially indicating a significant inflammatory phase prior to degeneration. For degenerative DDH, the bone metabolism biomarker SOST was increased in DDH & OA compared to non-degenerative DDH. This may indicate that there are significant boney changes when the pathology progresses from DDH to secondary OA. Furthermore, OC concentrations were decreased in those with secondary OA compared to their healthy-hip controls, indicating there may be boney metabolism differences related

to DDH. These results align with previous research highlighting the changes in subchondral bone with the progression of OA and the differential bone microstructure that is associated with DDH.[26,27] However, further research targeting mechanisms specific to bone and considering the effects of aging and activity is needed before conclusions can be made.

Significant differences in the concentrations of catabolic related (FGF-2), anabolic related (PDGF-AB/BB), inflammatory related (MCP-1 and MCP-3), cartilage extracellular matrix production (PIICP), and regulation of bone formation (OC) in the serum and urine of participants with degenerative DDH and secondary hip OA were observed when compared to similarly aged participants with healthy hips. As described previously, FGF-2 induces catabolic effects on human articular cartilage[13,14] but PDGF-AB/BB has not been well described with respect to OA. However, studies show that platelet rich plasma injection may cause an increase in serum PDGF-AB/BB which researchers believe may help aid in tissue regeneration.[28,29] Interestingly, MCP-1 and MCP-3 were unexpectedly decreased in the participants with degenerative OA compared to their healthy-hip controls. While there is minimal research published on MCP-3 and OA, in a meta-analysis, MCP-1 has been described to have increased serum concentrations in those with OA compared to controls.[30]

PIICP has been described as an early predictor for progression of OA.[31] PIICP is the end carboxyl of type II procollagen that is cleaved when it is integrated into tissue, and one study reported a positive correlation with knee synovial fluid PIICP concentrations and joint space narrowing at 4 years.[31] Interestingly, serum

COMP has previously been associated with knee osteoarthritis, and implicated in hip OA as well, but it did not differentiate degenerative DDH with secondary hip OA from cohort matched healthy-hip controls in the present study.[32,33] This suggests that hip osteoarthritis secondary to DDH may have unique mechanisms driving degenerative joint disease such that different serum and urine protein biomarkers will distinguish it from primary hip osteoarthritis.

When comparing biomarker concentrations in the control cohorts, there were age-related decreases in urinary MCP-1 and TIMP-1. A previous mouse study indicated that circulating MCP-1 was decreased with aging.[34] While we saw a decrease in TIMP-1 concentrations associated with aging, there have not been other studies that have investigated this so further validation is needed.

Taken together, these data provide insight into protein concentration differences in symptomatic DDH prior to and following the development of OA. Serum and urine protein biomarkers may have potential for defining clinically relevant stages of DDH and may point towards disease mechanisms in the development and progression of DDH to secondary OA. These results indicate that clinically applicable biomarkers for use in screening, diagnosis, and staging for DDH may be developed and validated using protein biomarker panels from serum and/or urine samples.

There are several limitations to this study that should be considered when interpreting and applying the results. The patient population studied was relatively small with a very limited number of subjects that were healthy-hip controls for the DDH & OA cohort. Although the patient population was predominantly female, this

is consistent with the epidemiology of DDH.[35–37] In addition, patient cohorts were considered only on age range of 13-34 and 35-54 years, such that potentially confounding factors including comorbidities, body mass index, medications, and others were not controlled for in patient inclusion or analyses. However, this experimental design was intentional such that the translational application of biomarker panels to 'real life' patient populations would be clear. Future studies should include a larger sample size to account for these limitations and conduct analyses which consider these confounders.

The results of the present study provide initial evidence for serum and urine protein biomarkers to characterize mechanisms of disease that define DDH and its progression to secondary hip osteoarthritis. Further clinical assessment of the discriminatory capabilities of these proteins may allow for the development and validation of panels for screening, diagnosis, and staging for DDH. Ongoing studies in our laboratory are using receiver operator characteristic curve analyses to assess biomarker panels based on similar proteins elucidated from the present study to determine the presence of DDH in patients from a blood or urine sample. With more effective screening methods for DDH, clinicians will be empowered to accurately diagnosis and stage DDH prior to irreversible pathology such that decision-making regarding type and timing of preventative and therapeutic interventions can be evidence based.

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Urine Related Biomarker Concentrations

		DDH	DDH & OA	DDH Controls	DDH & OA Controls
Anti-Degradative	TIMP-1	0.952 (IQR: 0.1660-4.525)	1.668 (IQR: 0.252-8.517)	2.405 **#** (IQR: 0.595-5.247)	0.374 (IQR: 0.114-0.554)
	TIMP-3	0.27 (IQR: 0.05-2.57)	8.69 ***** (IQR: 0.29-34.17)	0.71 (IQR: 0.09-3.40)	0.33 (IQR: 0.13-0.57)
Degradative	MMP-7	52.49 (IQR: 31.28-99.18)	174.99 ***** (IQR: 82.26-685.49)	33.97 (IQR: 19.67-66.87)	44.83 (IQR: 21.19-211.26)
Inflammatory	MCP-1	1.037 (IQR: 0.434-1.608)	1.408 (IQR: 0.434-3.224)	1.218 **#** (IQR: 0.364-11.330)	0.406 (IQR: 0.124-0.629)
Bone Metabolism	SOST	0.62 (IQR: 0.15-1.16)	1.48 ***** (IQR: 0.72-3.12)	2.16 (IQR: 0.21-6.39)	0.46 (IQR: 0.23-1.34)
Anabolism	PIICP	0.54 (IQR: 0.36-6.19)	30.76 **#** (IQR: 0.73-52.06)	0.30 (IQR: 0.23-0.51)	0.36 (IQR: 0.27-0.46)
	FGF	0.043 (IQR: 0.007-0.127)	0.24 **#** (IQR: 0.028-0.51)	0.008 (IQR: 0.003-0.014)	0 (IQR: 0-0.004)
	PDGF-AB/BB	0.018 (IQR: 0.01-0.11)	0.08 **#** (IQR: 0.007-0.62)	0.006 (IQR: 0.001-0.25)	0.004 (IQR: 0.001-0.007)

Table 7-1: Median urine biomarker concentrations (pg/mL/creatinine) and interquartile range (IQR) with statistical significance ($p<0.05$) from a Mann-Whitney U statistical analysis.
*****= significantly different over the DDH cohort.
#= significantly different over the DDH & OA control cohort.

Serum Related Biomarker Concentrations

		DDH	DDH & OA	DDH Controls	DDH & OA Controls
Inflammatory	IL-1RA	68.18 * (IQR: 37.60-10009.29)	9.69 (IQR: 6.29-17.99)	88.86 (IQR: 45.44-1547)	45.44 (IQR: 16.2-151.09)
	IL-4	170.32 * (IQR: 27.45-1764.75)	3.50 (IQR: 1.74-13.86)	31.9 (IQR:4.55-1084)	4.92 (IQR: 2.92-136)
	IL-6	9.3 * (IQR: 1.12-56.17)	0 (IQR: 0-0)	29.1 (IQR: 0-60.56)	0 (IQR: 19.72)
	IL-13	7.99 * (IQR: 1.55-95.26)	0 (IQR: 0-0.28)	58.35 (IQR: 0.17-112.90)	0.13 (IQR: 0-32.19)
	MCP-1	377.38 (IQR: 315.59-522.67)	342.74 (IQR: 310.36-482.22)	628.61 (IQR: 528.99-1415)	901.56 *# (IQR: 758.99-1002.52)
	MCP-3	184.26 * (IQR: 43.93-351.17)	8.03 (IQR: 4.79-21.46)	186.34 (IQR: 36.81-376.03)	51.22 * (IQR: 16.53-132.90)
Bone Metabolism	OC	7430 (IQR: 5313-13121)	8283 (IQR: 4145-9726)	22443 (IQR: 1092-40356)	17602 * (IQR: 14597-37221)
Anabolism	FGF	48.83 * (IQR: 28.63-67.44)	23.91 (IQR: 19.63-27.77)	43.06 (IQR: 27.10-89.01)	35.92 (IQR: 34.24-39.17)

Table 7-2: Median serum biomarker concentrations (pg/mL) with interquartile range (IQR) with statistical significance ($p<0.05$) from a Mann-Whitney U statistical analysis.
*****= significantly different over the DDH & OA cohort.
#= significantly different over the DDH control cohort.

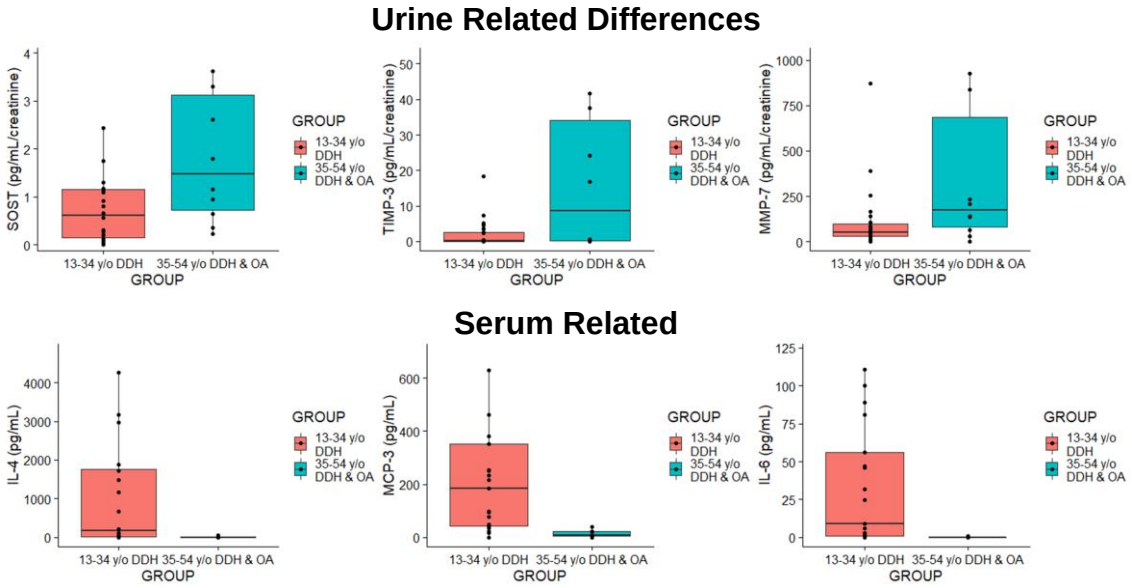

Figure 7-1: Significant urine and serum related differences in median protein concentrations between the DDH cohort and the DDH & OA cohort with median and IQR denoted by the middle and end points of the "box" in the boxplot.

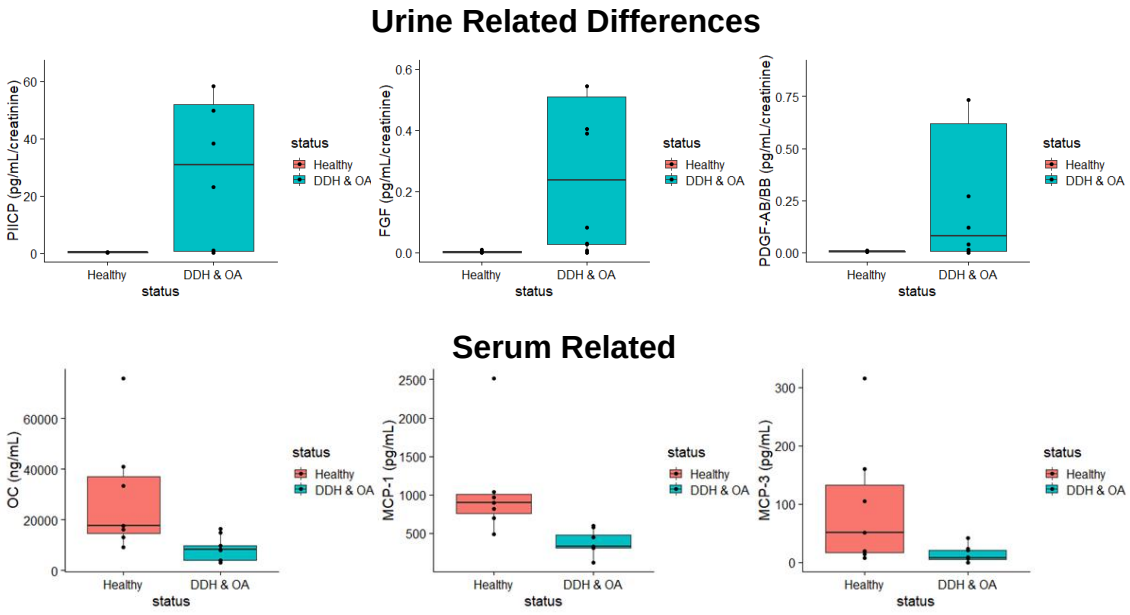

Figure 7-2: Significant urine and serum related median differences in protein concentrations between the DDH & OA cohort to the control for DDH & OA with median and IQR denoted by the middle and end points of the "box" in the boxplot.

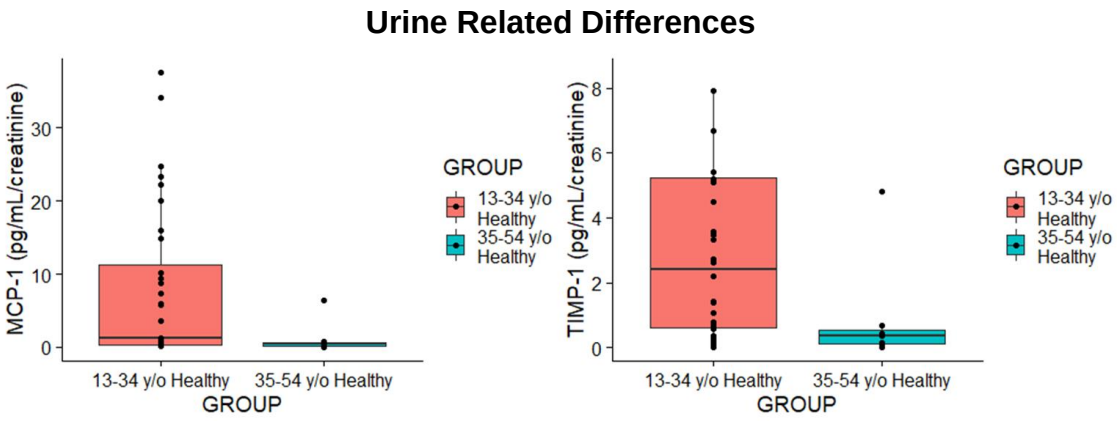

Figure 7-3: Significant urine related differences in protein concentrations between the DDH control cohort and DDH & OA control cohort with median and IQR denoted by the middle and end points of the "box" in the boxplot.

CHAPTER 8:

EVALUATION OF SERUM AND URINE BIOMARKERS FOR DEVELOPMENTAL DYSPLASIA OF THE HIP PRIOR TO ONSET OF SECONDARY OSTEOARTHRITIS

Research Objective and Hypothesis:

Developmental dysplasia of the hip (DDH) is a cause of early-onset hip osteoarthritis (OA).[1] The progression of DDH to hip OA can be mitigated when timing and accuracy of diagnosis allow for preventative interventions to be implemented effectively.[2–4] Current, routine diagnostics for DDH are associated with a misdiagnosis rate of at least 14%, such that hip OA secondary to DDH is a growing healthcare concern.[5] As such, improved methods for screening and early diagnosis for DDH are critically needed. Previous research has identified panels of serum and urine protein biomarkers to discriminate between dogs with healthy hips and those with hip dysplasia, prior to the onset of secondary osteoarthritis.[6] Based on these results and the extensive similarities between canine and human hip dysplasia, development and validation of serum and urine biomarker panels for human hip dysplasia merit investigation.[7,8] Protein biomarker panels that distinguish healthy-hip patients from those with DDH, prior to onset of secondary hip OA, would provide an objective, easy-to-obtain, and cost-effective method for avoiding misdiagnosis and improving patient outcomes.[9–12] Therefore, this study was designed to evaluate serum and urine biomarker panels for their capabilities in discriminating between healthy-hip and DDH status in 13-34 year-old patients.

It was hypothesized that one or more bodily fluid biomarker panels could effectively differentiate patients with DDH from patients with healthy-hips in a population at risk for developing secondary hip OA if not effectively diagnosed and treated.

Materials and Methods

Sample Collection:

With IRB approval (#2012192) and informed patient consent, blood and urine were collected from two cohorts of human subjects:

- DDH cohort (n=32): Young adult (13-34 years old) patients with physician confirmed DDH, prior to clinical or radiographic signs of secondary hip OA.
- Healthy-hip controls (n=35): 13–34-year-old volunteers with no clinical or radiographic signs of DDH.

Exclusion criteria included incompetent adults, prisoners, patients with cancer or received cancer treatment within the past 6 months, patients who had any surgery within the last 6 months, previous hip surgeries at any time, recent joint trauma, metabolic disorders, rheumatoid arthritis, corticosteroid injections within the last 6 months, currently taking oral corticosteroids, serious organ diseases or failures, are pregnant or lactating, or syndromic diseases (e.g. Cystic fibrosis or multiple sclerosis). DDH was determined based on examination by a board-certified orthopaedic surgeon in conjunction with diagnostic imaging assessments of the hips, including anteroposterior, Dunn, false-profile, and lateral

radiographic views. Computed tomography (CT) was utilized to determine the presence of acetabular version and/or femoral torsion. Measurements of left center-edge angle, anterior center-edge angle, Tönnis angle, acetabular extrusion index, alpha angle, head sphericity, acetabular version, and femoral torsion were also utilized for diagnosis. Hips were categorized by definitive diagnosis for the DDH groups and controls were self-reported as not having any previous hip pathologies.

Sample Collection and Storage

Blood and urine samples were collected from eligible participants at the same clinical location at the author's institution. Whole blood (2-6 ml) was collected by aseptic peripheral venipuncture in a Vacutainer Serum Tube (Becton, Dickinson and Company, Franklin Lakes, NJ) and urine (>4 ml) was collected by voluntary micturition. After sample collection, the fluids were immediately transported to an on-site laboratory for processing. Whole blood samples sat at room temperature for 30 minutes then were centrifuged (1200xg, 10 minutes), followed by serum collection. Serum and urine samples were aliquoted and stored at -80°C for subsequent analyses.

Protein Analysis

To measure dynamics of collagen degradation and synthesis, cross linked C-telopeptide of type I collagen (CTX-I), and cross linked C-telopeptide type II collagen (CTX-II), procollagen I C-terminal propeptide (PICP) and procollagen II

C-terminal propeptide (PIICP) using commercially available enzyme-linked immunosorbent assays (ELISA) assays (ABclonal; Woburn, MA). The concentration of hyaluronan (HA), a disintegrin-like and metalloproteinase domain with thrombospondin motifs 4 (ADAMTS4) and ADAMTS5, receptor activator of NF-kappa B ligand (RANKL), and Cartilage Oligomeric Matrix Protein (COMP) in the samples were assessed using DuoSet ELISA assays according to the manufacturer's protocol (R&D Systems; Minneapolis, MN). The concentration of matrix metalloproteinase (MMP)-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-13 was determined using Human multiplex Luminex immunoassay (R&D System, Minneapolis. MN). The concentration of dickkopf-related protein 1 (DKK-1), osteoprotegrin (OPG), osteocalcin (OC), osteopontin (OPN), sclerostin (SOST), and parathyroid hormone (PTH) using the Human Bone Metabolism multiplex Luminex immunoassay (MilliporeSigma, Burlington, MA). The concentration of fibroblast growth factor (FGF)-2, Fractalkine, interferon (IFN)-γ, growth-regulated oncogene (GRO)-α, interleukin (IL)-1β, IL-1-receptor antagonist(RA), IL-4, IL-6, IL-10, and IL-13, monocyte chemoattractant protein (MCP)-1 and MCP-3, platelet derived growth factor (PDGF)-AA and PDGF-AB/BB, macrophage inflammatory protein (MIP)-1α and MIP-1β, regulated upon activation, normal T expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF) using the Human Cytokine/Chemokine multiplex Luminex immunoassays (MilliporeSigma, Burlington, MA). The concentration of tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, TIMP-3, and TIMP-4 was determined using the Human TIMP

multiplex Luminex immunoassay bead panel 2 (MilliporeSigma, Burlington, MA). The urine creatinine concentration was measured with the creatinine colorimetric assay (Cayman Chemical Company; Ann Arbor, MI), and was used to standardize the urinary concentrations obtained for the other assays.

Statistical Analysis:

Data were summarized and non-normality was determined using Shapiro-Wilk normality tests. The data were not normally distributed, so a Mann-Whitney U test was performed to determine significant differences in the serum and urine biomarker concentrations between patients with diagnosed DDH and their healthy controls. Significance was determined by a two-sided $p<0.05$ and Bonferroni corrections for multiple comparisons were made.

Discernably different biomarkers with the largest median differences were considered for the biomarker panels. Panels were first created within biomarker types (e.g., inflammatory/anabolism, bone-related, degradative) for each fluid type, the top four biomarkers for each fluid type, and a combined fluid panel with two top biomarkers from each fluid type. The biomarkers with the lowest p-values for each grouping of biomarkers were hand selected and utilized in a logistic regression model with DDH as the outcome of interest (e.g., general linear model using the log link function). A Hosmer-Lemeshow test was used to determine model overfitting, with $p<0.05$ indicating overfitting. Logistic regression models were used for ROC curve analyses and the area under the curve (AUC) obtained. The optimal threshold for each ROC curve was calculated to maximize, while equally weighting,

both sensitivity and specificity using a previously described process.[13] All analyses were performed in R version 4.1.2 (R Core Team, 2021), figures were produced using the ggplot2 package (v3.3.5; Wickham H, 2016), and ROC analysis using pROC (v1.18.0; Robin X, 2021).

Results:

Differences in Protein Biomarker Concentrations Related to Hip Status:

In the urine **(Table 8-1)**, DDH patients had significantly increased median concentrations of PIICP (p=0.037) **(Figure 8-1A)**, FGF-2 (p=0.03) **(Figure 8-1B)**, IL-10 (p=0.037) **(Figure 8-1C)**, IL-1RA (p=0.002) **(Figure 8-1D)**, and MIP-1β (p=<0.001) **(Figure 8-1E),** compared to healthy controls.

In the serum **(Table 8-2 & 8-3)**, DDH patients had significantly decreased median concentrations of DKK-1 (p=0.003) **(Figure 8-2A)**, OC (p<0.001) **(Figure 8-2B)**, SOST (p<0.001) **(Figure 8-2C)**, MMP-8 (p=0.003) **(Figure 8-2D)**, TIMP-3 (p=0.02) **(Figure 8-2E)**, TIMP-4 (p=0.049) **(Figure 8-2F)**, MCP-1 (p<0.001) **(Figure 8-2G)**, RANTES (p<0.001) **(Figure 8-2H)**, GRO-α (p=0.007) **(Figure 8-2I)**, and PDGF-AA (p=0.004) (**Figure 8-2J)** compared to healthy controls.

All other biomarkers were not significantly different between groups.

Diagnostic Biomarker Panels:

The following panels are listed with the biomarkers contained within them, their AUC, H-L value, sensitivity, and specificity. **(Table 8-4)** A urinary inflammatory related biomarker panel **(Figure 8-3A)** including IL-10, IL-1RA, and MIP-1β had

an AUC of 0.726 (CI: 0.565-0.886) with no evidence of overfitting and a specificity of 1.000 and sensitivity of 0.542. A urinary panel with the top four significantly different biomarkers **(Figure 8-3B)** including PIICP, FGF, IL-1RA, and MIP-1β had an AUC of 0.708 (CI: 0.531-0.884) with no evidence of overfitting and a specificity of 0.895 and sensitivity of 0.500.

A serum bone metabolism related biomarker panel **(Figure 8-4A)** including DKK-1, OC, and SOST had an AUC of 0.809 (CI: 0.692-0.925) with no evidence of overfitting and a specificity of 0.800 and sensitivity of 0.739. A serum degradative enzyme related biomarker panel **(Figure 8-4B)** including MMP-8, TIMP-3, TIMP-4 had an AUC of 0.734 (CI:0.601-0.686) with an no evidence of overfitting and a specificity of 0.606 and sensitivity of 0.905. A serum chemokine and growth factor biomarker panel **(Figure 8-5A)** including GRO-α, PDGF-AA, MCP-1, and RANTES had an AUC of 0.824 (CI: 0.712-0.936) with no evidence of overfitting and a specificity of 0.727 and sensitivity of 0.857. A serum panel with the top four significantly different biomarkers **(Figure 8-5B)** including DKK-1, OC, MCP-1, and RANTES had an AUC of 0.817 (CI: 0.706-0.928) with no evidence of overfitting and a specificity of 0.706 and sensitivity of 0.928.

A panel including the top two significantly different urine (IL-1RA and MIP-1β) and serum (RANTES and OC) biomarkers **(Figure 8-6)** had an AUC of 0.959 (CI: 0.903-1.0) with no evidence of overfitting and a specificity of 1.000 and sensitivity of 0.850.

Discussion:

The results from this study support the hypothesis that one or more bodily fluid biomarker panels could effectively differentiate patients with DDH from healthy-hip controls in a population at risk for developing secondary hip OA. With the exception of the urine combined panel, the combined panels that were fluid-specific and biomarker grouping-specific were able to provide good to excellent discrimination between individuals with healthy hips and those with DDH. Interestingly, a panel containing two urine biomarkers and two serum biomarkers with the most statistically significant differences in concentrations between cohorts provided the greatest discrimination of hip status based on its sensitivity of 0.85 and specificity of 1. Based on relative ease and standard-of-care for sample collection, in conjunction with the rapid method of analysis, this combined-sample four-biomarker panel could be easily incorporated into clinical practice as a screening tool for detection of DDH prior to the onset of secondary hip OA in this at-risk patient population. However, prospective longitudinal studies assessing a larger and more diverse population of patients are required to validate this biomarker panel for clinical application.[14]

When analyzing the differences in urine biomarkers, DDH patients had increased urinary concentrations of type II collagen production (PIICP), catabolism (FGF-2), and inflammation (IL-1RA, IL-10, and MIP-1β) related proteins compared to controls. Higher levels of IL-1RA and IL-10 in the urine of dysplastic patients suggest an attempted regulation of inflammatory processes in DDH in an attempt to counteract its progression to hip OA as both of these ILs have been described as potential targeted treatments against the progression of OA.[15–18] MIP-1β, a

macrophage recruiting protein, has been described in previous research as being increased by articular chondrocytes in response to stimulation with IL-1β and has been correlated to severity of knee OA with indications that MIP-1β may increase tissue inflammation.[19–21] FGF-2 has been described as acting on FGFR which has a complex cascade following the activation by FGF-2 and *in vitro* research has indicated that FGF-2 induces catabolic effects on human articular cartilage.[22,23] In conjunction with these inflammatory processes, articular chondrocytes often upregulate collagen production for tissue repair and remodeling processes which has been increased in early OA and subsequently decreased in the late-stage OA, explaining the increases in the type II procollagen cleavage product, PIICP, in this population of DDH patients that have not yet progressed to OA.[24,25] These results may indicate that in this DDH patient population there is an increase in catabolism and concurrent tissue repair response by upregulating collagen production and reducing catabolic effects through anti-inflammatory mechanisms.

When analyzing differences in serum biomarkers, young adult DDH patients had decreased serum concentrations of bone metabolism-related (DKK-1, OC, and SOST), degradative (MMP-8, TIMP-3, and TIMP-4), inflammatory (GRO-α, MCP-1, and RANTES), and anabolism-related (PDGF-AA) proteins compared to controls. Lower levels of the bone metabolism biomarkers, DKK-1, OC, and SOST, may be indicative of the altered bone development that is evident in the phase of DDH prior to the presence of OA.[26–30] This reduction in bone metabolism may be the result of a response to the differential joint loading associated with DDH or other factors that have not been fully elucidated.[31,32] The decrease in PDGF-AA

supports this latter hypothesis in that previous studies have reported that PDGF-AA can promote bone formation and healing by guiding osteogenic differentiation of mesenchymal stem cells, so this decrease may be correlated with the decrease in the acetabular development laterally to cover the femoral head in DDH.[33–35] Interestingly, DDH patients had decreased serum concentrations of degradation-related MMP-8, TIMP-3, and TIMP-4. The decrease in MMP-8 runs counterintuitive to the idea that these patients are progressing toward degenerative joint disease, but follows what we would expect with TIMPs decreasing as patient progress to OA.[36–38] Similarly, the decreased serum concentrations of inflammatory proteins, GRO-α, MCP-1, and RANTES, in DDH patients is counterintuitive to the idea that inflammatory responses are increased when the joint is dysplastic.[39–42]

For the goals of this research, sensitivity should be prioritized for a low false negative rate to reduce misdiagnosis while capturing all potential positive cases for further assessment and monitoring while still attempting to keep a high specificity to reduce false positives. As such, the results of this experiment favor the combined serum panel for further assessment towards clinical use as it had a sensitivity of 0.85 and a specificity of 1.0 while the serum combined panel would still be of interest as it had a slightly higher sensitivity of 0.93 but had a reduced specificity of 0.71.

There are several limitations to this study that should be considered when interpreting and applying the results. The number of subjects in each cohort was relatively small and only the age ranges for cohorts were aligned. As such, potentially confounding variables including race, body mass index, occupation,

activities, comorbidities, medications, and others were not controlled for in the experimental design or analyses. However, this “all-comers” approach was intentional based on the goal for translational application of DDH biomarker panels to ‘real life’ patient populations. In addition, the study involved a cross-sectional analysis of a limited age range of individuals such that the biomarker panels cannot be considered to have mechanistic or prognostic implications. The panels developed in this study were not tested against an independent data set, and the study only include patients with physician diagnosed DDH. Therefore, it is not known if the developed panels will have the same sensitivity and specificity characteristics when assessed on an independent patient population, or in a patient population where DDH is undiagnosed and asymptomatic. Future studies should include longitudinal sampling in a larger population of participants over a broad demographic spectrum with correlation of biomarker data to clinical, diagnostic, and functional measures of disease severity and progression.

The results of the present study provide initial proof-of-concept data that indicate panels of protein biomarkers measured in the urine and serum may be able to differentiate young adults with non-degenerative DDH from young adults without a DDH diagnosis. Of the panels developed and assessed, a panel of two serum and two urinary proteins provided the best combined sensitivity and specificity results while the combined serum panel provided the maximal sensitivity which may be more ideal for clinical implementation. Taken together, these data suggest that there is potential for a non-invasive method for cost-effective and timely screening for discriminating between DDH in this at-risk population from

healthy-hip controls. Further development and validation of these biomarker panels may result in highly sensitive and specific tools for early diagnosis, staging, and prognostication of DDH, as well as treatment decision making and monitoring capabilities filling a critical unmet need in healthcare.

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	DDH Cohort	Healthy Hip Controls	Median Difference (Healthy - DDH)	p-value
IL-1RA (pg/mL/creatinine)	1.4314 (IQR: 0.5124-15.7816)	0.1011 (IQR: 0.0280-0.6420)	-1.3303	**0.0015**
IL-1β (pg/mL/creatinine)	0.0019 (IQR: 0.0005-0.0064)	0.0007 (IQR: 0.0003-0.0008)	-0.0012	0.0675
IL-4 (pg/mL/creatinine)	0.0041 (IQR: 0.0008-0.020)	0.0020 (IQR: 0.0010-0.0046)	-0.0021	0.5821
IL-10 (pg/mL/creatinine)	0.0004 (IQR: 0.0-0.0012)	0 (IQR: 0.0-0.0004)	-0.0004	**0.0374**
IFNγ (pg/mL/creatinine)	0.0001 (IQR: 0.0-0.0017)	0 (IQR: 0.0-0.0004)	-0.0001	0.2689
GRO-α (pg/mL/creatinine)	0.0961 (IQR: 0.0226-0.2513)	0.0183 (IQR: 0.0035-0.3721)	-0.0779	0.1080
Fractalkine (pg/mL/creatinine)	0.1312 (IQR: 0.0234-0.2510)	0.0682 (IQR: 0.0418-0.0935)	-0.0630	0.2594
MCP-1 (pg/mL/creatinine)	1.0041 (IQR: 0.4338-1.6085)	1.2176 (IQR: 0.3644-11.3300)	0.2135	0.2529
MCP-3 (pg/mL/creatinine)	0.0044 (IQR: 0.0003-0.0335)	0.0023 (IQR: 0.0-0.0045)	-0.0021	0.2764
MIP-1β (pg/mL/creatinine)	0.0016 (IQR: 0.0-0.0060)	0 (IQR: 0.0-0.0)	-0.0016	**<0.0001**
RANTES (pg/mL/creatinine)	0.0340 (IQR: 0.0138-0.2909)	0.0175 (IQR: 0.0067-0.3830)	-0.0165	0.2252
PDGF-AA (pg/mL/creatinine)	0.1397 (IQR: 0.0950-0.5812)	0.4728 (IQR: 0.0697-3.1428)	0.3331	0.3381
FGF-2 (pg/mL/creatinine)	0.0430 (IQR: 0.0073-0.1273)	0.0076 (IQR: 0.0033-0.0139)	-0.0354	**0.0302**
VEGF (pg/mL/creatinine)	0.0378 (IQR: 0.0192-0.2419)	0.0247 (IQR: 0.0100-0.0562)	-0.0131	0.3590
DKK-1 (pg/mL/creatinine)	0 (IQR: 0.0-0.1131)	0.1040 (IQR: 0.0-0.4957)	0.1040	0.1897
OPG (pg/mL/creatinine)	0.0238 (IQR: 0.0137-0.0435)	0.0660 (IQR: 0.0148-0.7917)	0.0422	0.1669
OC (pg/mL/creatinine)	5.1774 (IQR: 1.6334-119.0589)	43.8298 (IQR: 2.2596-185.2374)	38.6524	0.1611
OPN (pg/mL/creatinine)	35.3461 (IQR: 9.3834-236.3738)	143.4683 (IQR: 23.3865-736.5068)	108.1222	0.0883
SOST (pg/mL/creatinine)	0.6695 (IQR: 0.1505-1.1565)	2.1553 (IQR: 0.2067-6.3917)	1.4858	0.0722
CTX-I (pg/mL/creatinine)	0.0028 (IQR: 0.0008-0.0080)	0.0044 (IQR: 0.0030-0.0079)	0.0016	0.1154
CTX-II (pg/mL/creatinine)	0.2075 (IQR: 0.1468-0.6836)	0.1848 (IQR: 0.1013-0.2636)	-0.0227	0.4264
PICP (pg/mL/creatinine)	0 (IQR: 0.0.-0.0023)	0.0019 (IQR: 0.0012-0.0022)	0.0019	0.0781
PIICP (pg/mL/creatinine)	0.5366 (IQR: 0.3591-6.1942)	0.2966 (IQR: 0.2342-0.5094)	-0.2399	**0.0363**
HA (ng/mL/creatinine)	0.0091 (IQR: 0.0049-0.0161)	0.0098 (IQR: 0.0066-0.0202)	0.0007	0.3370
MMP-2 (pg/mL/creatinine)	4.3974 (IQR: 1.4487-6.1927)	3.7497 (IQR: 2.8407-7.4944)	-0.6477	0.5296
MMP-3 (pg/mL/creatinine)	0.3661 (IQR: 0.2932-0.3957)	0.3410 (IQR: 0.1185-0.5299)	-0.0251	0.8626
MMP-7 (pg/mL/creatinine)	46.7707 (IQR: 31.2792-99.1801)	33.9748 (IQR: 19.6728-66.8677)	-12.7959	0.1121
MMP-8 (pg/mL/creatinine)	1.1329 (IQR: 0.1754-10.2211)	1.1727 (IQR: 0.1302-22.8384)	0.0399	0.8306
MMP-9 (pg/mL/creatinine)	6.0376 (IQR: 0.3580-44.9354)	5.5518 (IQR: 0.3067-65.6303)	-0.4858	0.9374
TIMP-1 (pg/mL/creatinine)	0.7349 (IQR: 0.1601-4.5247)	2.4051 (IQR: 0.5947-5.2474)	1.6702	0.1967
TIMP-2 (pg/mL/creatinine)	3.0407 (IQR: 1.7237-23.3692)	11.8543 (IQR: 1.3917-51.2011)	8.8136	0.5470
TIMP-3 (pg/mL/creatinine)	0.2607 (IQR: 0.0457-2.5692)	0.7103 (IQR: 0.0930-3.3980)	0.4496	0.3917
TIMP-4 (pg/mL/creatinine)	0.0005 (IQR: 0.0-0.0123)	0.0029 (IQR: 0.0-0.0104)	0.0024	0.6741

Urine Related Biomarker Concentrations

Table 8-1: Median urine biomarker concentrations and interquartile ranges. P-values are from a Mann-Whitney U test with Bonferroni correction.

	DDH Cohort	Healthy Hip Controls	Median Difference (Healthy - DDH)	p-value
IL-1RA (pg/mL)	68.18 (IQR: 37.60-1009.29)	88.86 (IQR: 45.44-1547)	20.69	0.9635
IL-1β (pg/mL)	2.06 (IQR: 0.70-3.39)	1.22 (IQR: 0.54-3.30)	-0.84	0.7269
IL-4 (pg/mL)	170.32 (IQR: 27.45-1764.75)	31.90 (IQR: 7.55-1084)	-138.42	0.2695
IL-6 (pg/mL)	9.30 (IQR: 1.12-56.17)	29.10 (IQR: 0.0-60.56)	19.80	0.8013
IL-10 (pg/mL)	4.72 (IQR: 1.62-13.36)	1.87 (IQR: 0.0-3.83)	-2.85	0.0853
IL-13 (pg/mL)	7.99 (IQR: 1.55-95.26)	58.35 (IQR: 0.14-112.9)	50.36	0.8726
IFNγ (pg/mL)	1.27 (IQR: 0.0-2.65)	1.07 (IQR: 0.47-7.23)	-0.20	0.6240
GRO-α (pg/mL)	1130 (IQR: 771.93-1614)	2159 (IQR: 1182-3764)	1029	**0.0062**
Fractalkine (pg/mL)	82.25 (IQR: 61.95-127.91)	82.26 (IQR: 64.67-154.16)	0.01	0.7105
MCP-1 (pg/mL)	377.38 (IQR: 315.59-522.67)	628.61 (IQR: 528.99-1415)	251.23	**0.0009**
MCP-3 (pg/mL)	184.26 (IQR: 43.93-351.17)	186.34 (IQR: 36.81-376.03)	2.08	0.9579
MIP-1α (pg/mL)	2.27 (IQR: 0.0-7.87)	0 (IQR: 0.0-21.45)	-2.27	0.7633
MIP-1β (pg/mL)	15.91 (IQR: 9.71-24.40)	38.06 (IQR: 12.06-89.51)	22.15	0.0836
RANTES (pg/mL)	2561 (IQR: 1997-4424)	6643 (IQR: 4574-12913)	4082	**<0.0001**
TNF-α (pg/mL)	3.34 (IQR: 1.47-4.97)	7.20 (IQR: 2.42-22.18)	3.86	0.0524
RANKL (pg/mL)	69.24 (8.46-174.01)	205.03 (IQR: 23.73-471.65)	135.79	0.4975
PDGF-AA (pg/mL)	1621 (IQR: 948.12-2234)	2737 (IQR: 2129-4119)	1116	**0.0040**
PDGF-AB/BB (pg/mL)	13046 (IQR: 10415-14736)	14332 (IQR: 12360-15829)	1286	0.0731
FGF-2 (pg/mL)	48.83 (IQR: 28.63-67.44)	43.06 (IQR: 27.10-89.01)	-5.77	0.8549
VEGF (pg/mL)	35.39 (IQR: 11.69-80.33)	53.91 (IQR: 16.50-110.12)	18.52	0.3685
DKK-1 (pg/mL)	498.86 (IQR: 304.83-752.37)	1611 (IQR: 622.96-4586.5)	1112.14	**0.0001**
OC (pg/mL)	7430 (IQR: 5313-13121)	22443 (IQR: 10922-40356)	15013	**0.0001**

Serum Related Biomarker Concentrations

Table 8-2: Median serum biomarker concentrations and interquartile ranges. P-values are from a Mann-Whitney U test with Bonferroni correction.

	DDH Cohort	Healthy Hip Controls	Median Difference (Healthy - DDH)	p-value
OPN (pg/mL)	1478 (IQR: 1154-3076)	2202 (IQR: 1258-8049)	724	0.2209
SOST (pg/mL)	577.92 (IQR: 471.40-733.10)	1542 (IQR: 796.32-2910)	964.08	**0.0007**
PTH (pg/mL)	19.79 (IQR: 11.03-37.57)	30.87 (IQR: 18.29-67.91)	11.08	0.2459
CTX-I (pg/mL)	4.35 (IQR: 1.85-12.12)	3.10 (IQR: 1.80-8.30)	-1.25	0.6966
CTX-II (pg/mL)	74.42 (IQR: 0.0-114.44)	72.01 (IQR: 0.0-101.61)	-2.41	0.5607
PICP (pg/mL)	6.75 (IQR: 2.22-25.99)	2.29 (IQR: 1.55-8.88)	-4.46	0.0781
PIICP (pg/mL)	190.88 (IQR: 84.40-2098)	86.43 (IQR: 75.48-203.75)	-104.45	0.1682
HA (ng/mL)	14.53 (IQR: 6.41-76.06)	12.71 (IQR: 8.24-20.00)	-1.82	1.0000
COMP (pg/mL)	1337.08 (IQR: 894-2038)	2071.14 (IQR: 329.64-6748)	734.06	0.7113
ADAMTS4 (pg/mL)	232.74 (IQR: 115.28-430.18)	350.09 (IQR: 289.00-826.74)	117.35	0.2776
ADAMTS5 (pg/mL)	0 (IQR: 0.0-731.06)	1204.05 (IQR: 485.89-2599)	1204.05	0.1123
MMP-1 (pg/mL)	250.65 (IQR: 132.09-1384)	703.49 (IQR: 190.36-1082)	452.84	0.7265
MMP-2 (pg/mL)	53574 (IQR: 42093-88549)	52217 (IQR: 38771-63472)	-1357	0.2822
MMP-3 (pg/mL)	5462 (IQR: 3768-14494)	6761 (IQR: 3648-12255)	1299	0.6027
MMP-7 (pg/mL)	5547 (IQR: 1005-7426)	1352 (IQR: 745.76-5656)	-4195	0.0831
MMP-8 (pg/mL)	86.35 (IQR: 0.0-167.53)	255.82 (IQR: 107.79-781.67)	169.47	**0.0034**
MMP-9 (pg/mL)	6206 (IQR: 2319-18213)	12108 (IQR: 5663-19056)	5902	0.3402
MMP-13 (pg/mL)	411.68 (IQR: 0.0-853-97)	50.09 (IQR: 0.0-695.14)	-361.59	0.2276
TIMP-1 (pg/mL)	10583 (IQR: 5827-38400)	14890 (IQR: 11762-21916)	4307	0.6068
TIMP-2 (pg/mL)	6945 (IQR: 5256-22545)	15656 (IQR: 7116-22455)	8711	0.1270
TIMP-3 (pg/mL)	1130 (IQR: 522.20-3297)	4000 (IQR: 1090-25526)	2870	**0.0226**
TIMP-4 (pg/mL)	225.81 (IQR: 121.66-341.37)	366.89 (IQR: 162.93-813.52)	141.08	**0.0499**

Serum Related Biomarker Concentrations cont.

Table 8-3: Median serum biomarker concentrations and interquartile ranges. P-values are from a Mann-Whitney U test with Bonferroni correction.

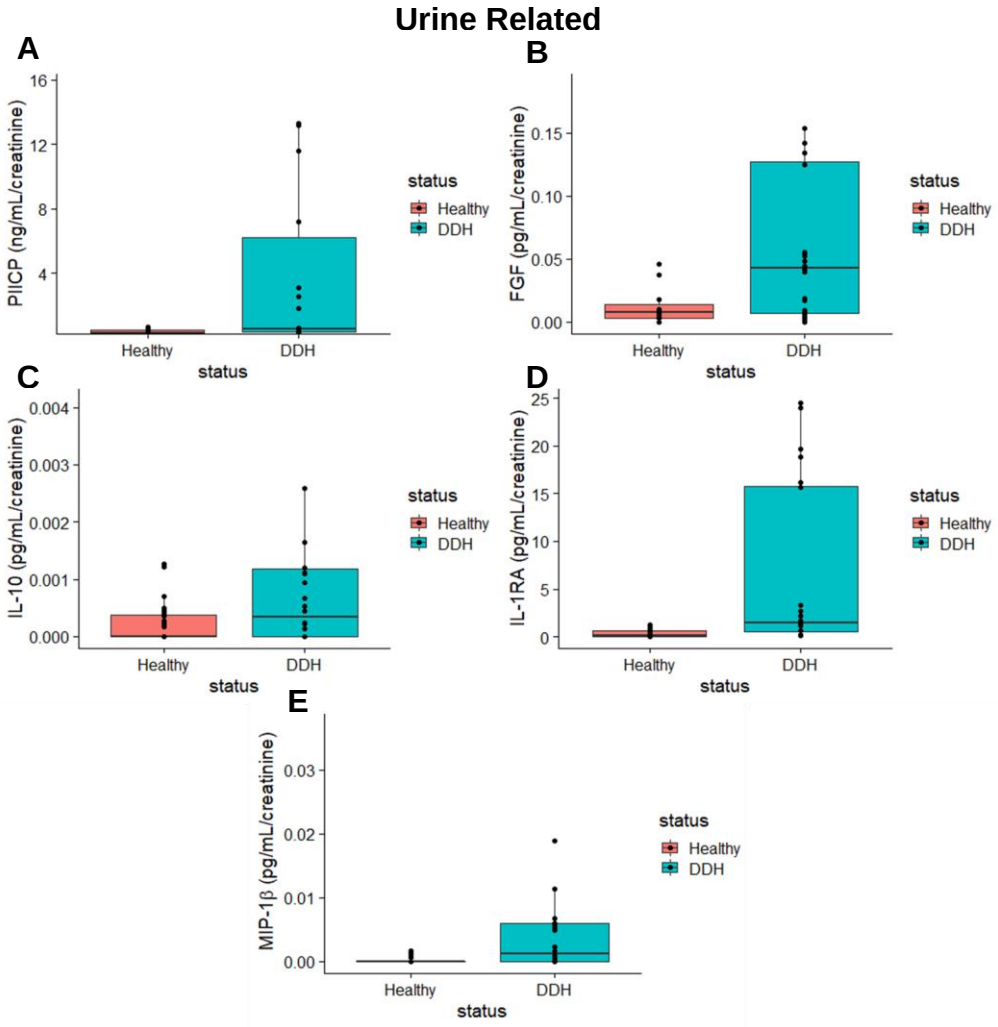

Figure 8-1: Boxplots of significantly different median urine biomarker concentrations between the DDH cohort and healthy controls ($p<0.05$). Median and the interquartile range are denoted by the middle and end points, respectively, of the "box" in the boxplot.

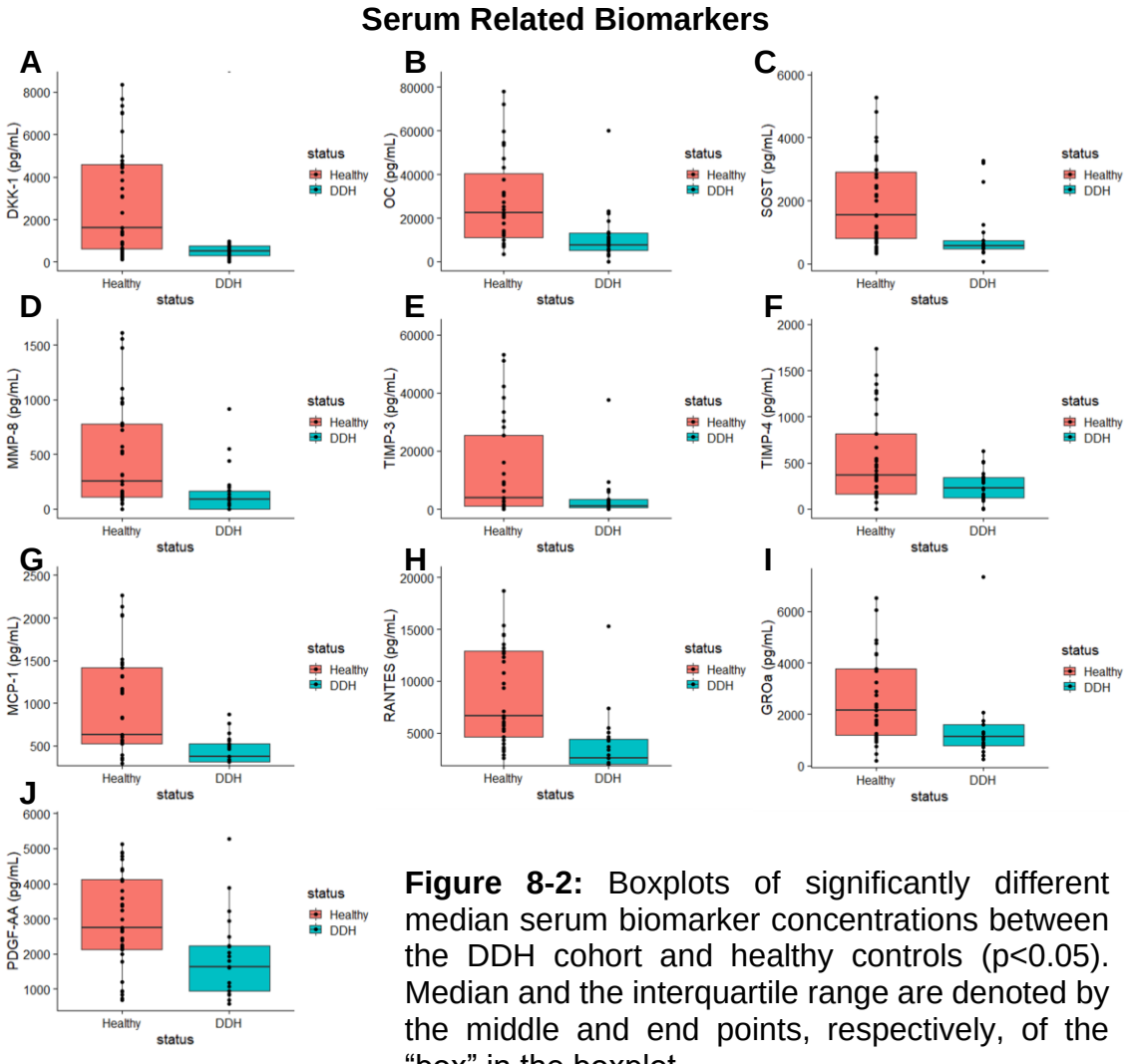

Figure 8-2: Boxplots of significantly different median serum biomarker concentrations between the DDH cohort and healthy controls ($p<0.05$). Median and the interquartile range are denoted by the middle and end points, respectively, of the "box" in the boxplot.

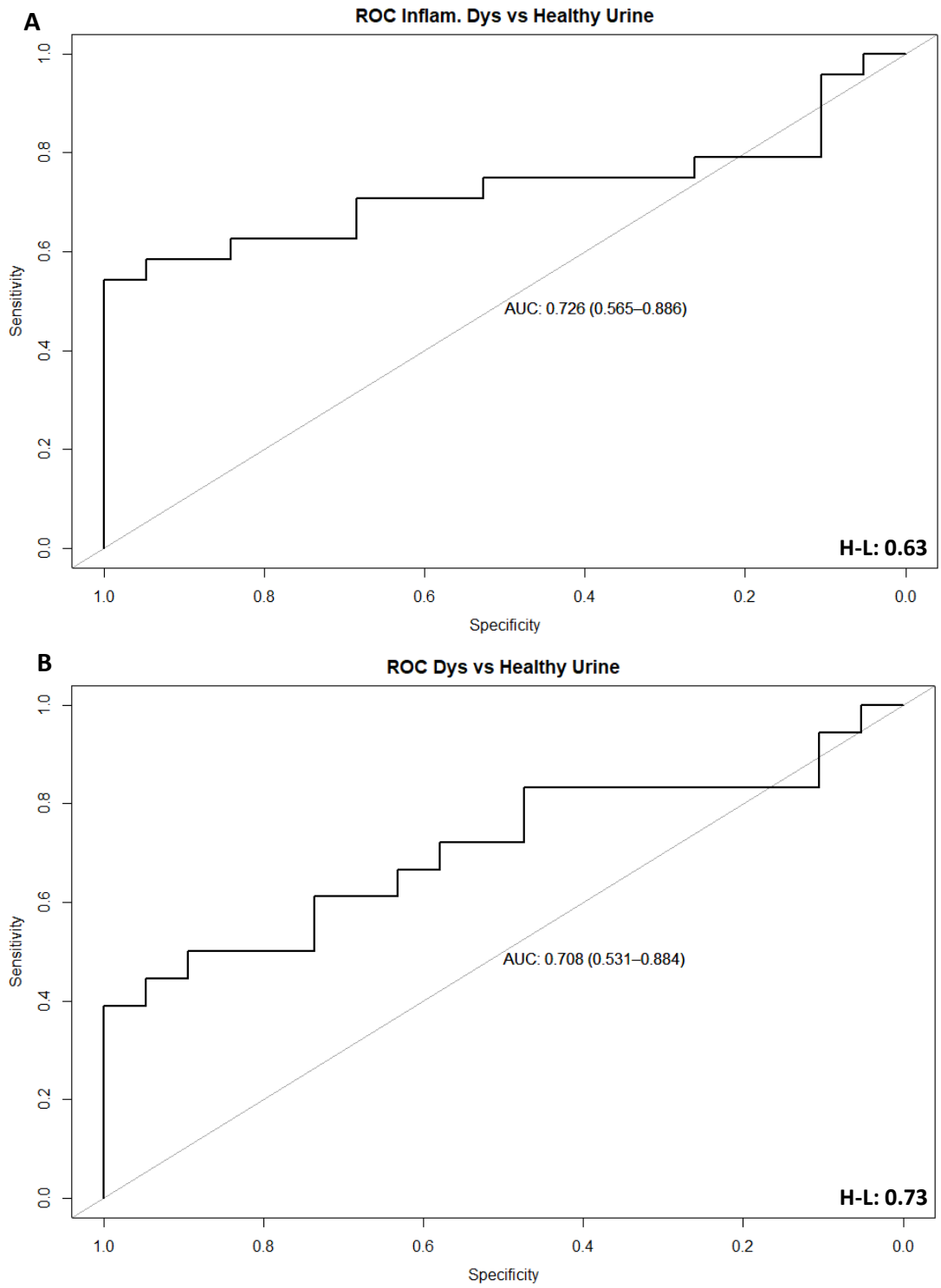

Figure 8-3: Urine related ROC curves containing A) inflammatory (Inflam.) related IL-10, IL-1RA, and MIP-1β and B) the combined panel containing PIICP, FGF, IL-1RA, and MIP-1β. Goodness of fit was determined with a Hosmer-Lemeshow test (H-L).

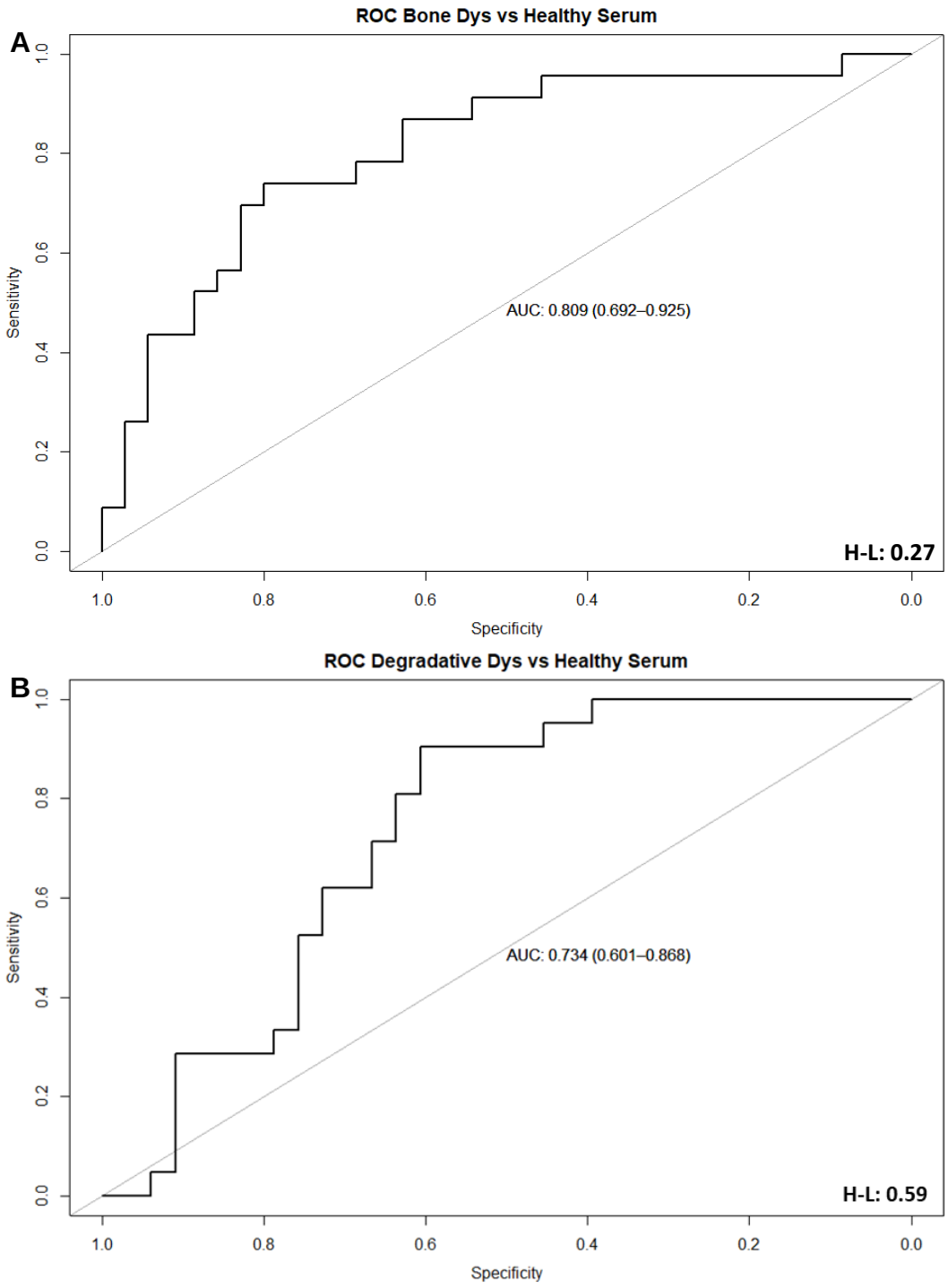

Figure 8-4: Serum related ROC curves containing A) bone related DKK-1, OC, and SOST and B) the degradative related panel containing MMP-8, TIMP-3, and TIMP-4. Goodness of fit was determined with a Hosmer-Lemeshow (H-L) test.

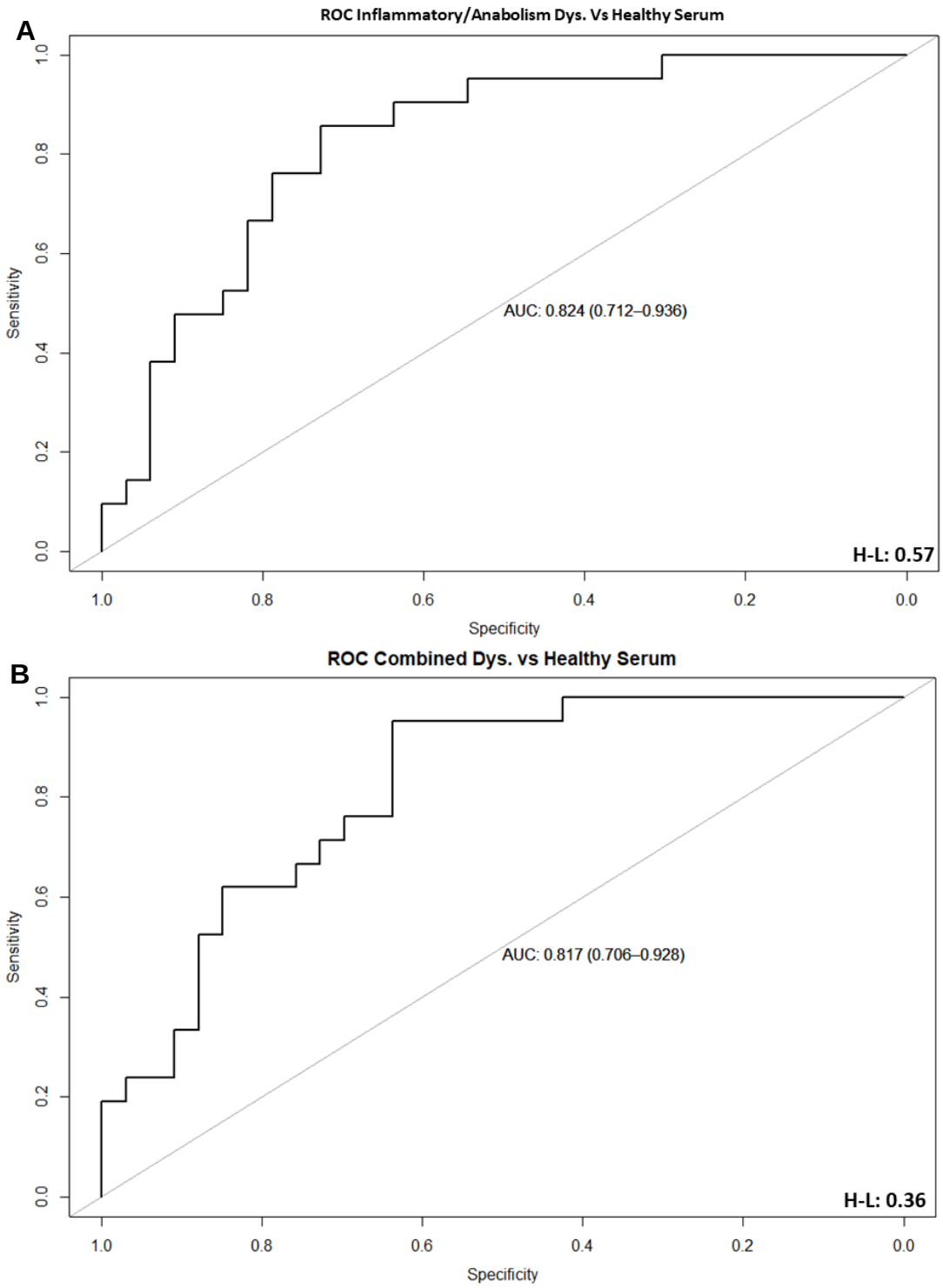

Figure 8-5: Serum related ROC curves containing A) inflammatory/anabolism related PDGF-AA, GRO-α, MCP-1, and RANTES, and B) the combined panel containing DKK-1, OC, MCP-1 and RANTES. Goodness of fit was determined with a Hosmer-Lemeshow (H-L) test.

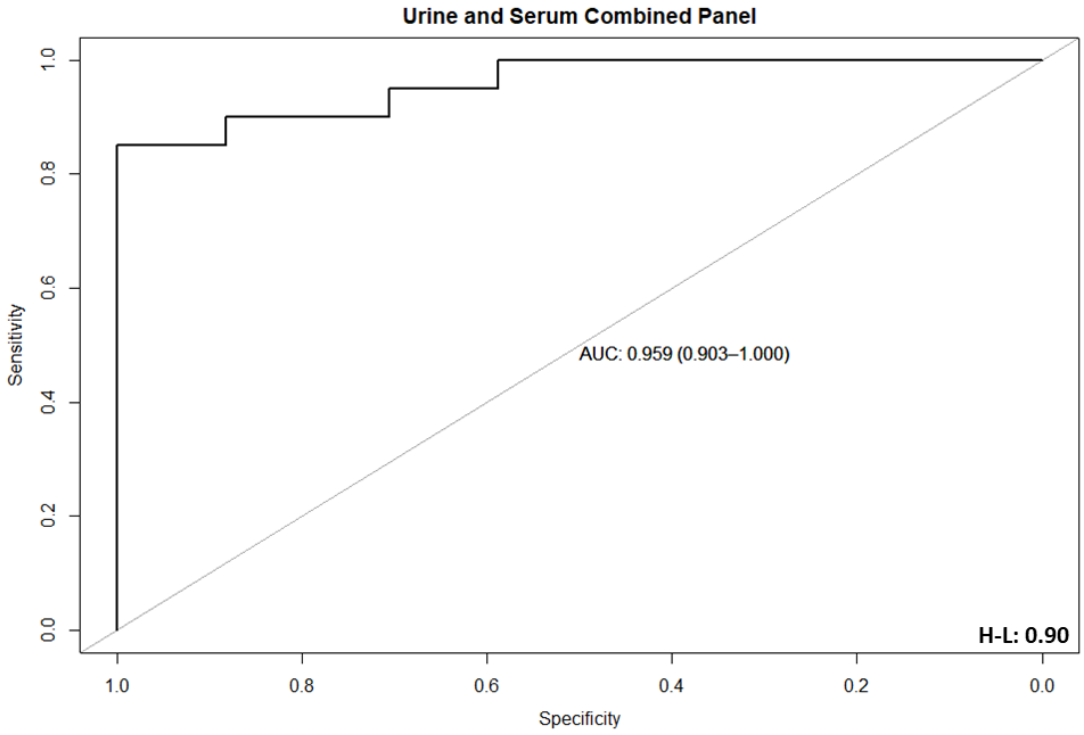

Figure 8-6: Urine and serum combined panel containing urine IL-1RA and MIP-1β along with serum RANTES and OC. Goodness of fit was determined with a Hosmer-Lemeshow (H-L) test.

Biomarker Panel Results

	Biomarkers	AUC	H-L	Specificity	Sensitivity
Urine Inflammatory	IL-10, IL-1RA, MIP-1β	0.726 (CI: 0.565-0.886)	0.63	1	0.542
Urine Combined	PIICP, FGF, IL-1RA, MIP-1β	0.708 (CI: 0.531-0.884)	0.73	0.895	0.500
Serum Bone Metabolism	DKK-1, OC, SOST	0.809 (CI: 0.692-0.925)	0.27	0.800	0.739
Serum Degradative	MMP-8, TIMP-3, TIMP-4	0.734 (CI: 0.601-0.868)	0.59	0.606	0.905
Serum Anabolism/ Inflmmatory	GRO-α, PDGF-AA, MCP-1, RANTES	0.824 (CI: 0.713-0.936)	0.57	0.727	0.857
Serum Combined	DKK-1, OC, MCP-1, RANTES	0.817 (CI: 0.706-0.928)	0.36	0.706	0.928
Serum and Urine Combined	Urine: IL-1RA, MIP-1β Serum: RANTES, OC	0.959 (CI: 0.903-1.000)	0.90	1	0.850

Table 8-4: Biomarker panels with their respective biomarker makeup, area under the curve (AUC), Hosmer-Lemeshow goodness of fit (H-L), and threshold for specificity and sensitivity.

CONCLUSIONS AND FUTURE DIRECTIONS

Developmental dysplasia of the hip (DDH) is a multifactorial disease that is potentially preventable and often treatable. Treatment largely addresses symptoms and works to mitigate progression to secondary osteoarthritis (OA). Prevention and treatment options are dependent on age and accuracy of diagnosis, which are currently based on subjective measures that often result in missed or misdiagnosis. To improve screening and early diagnosis methods for DDH, the pathobiological mechanisms associated with this disorder must be further characterized. Therefore, this dissertation research investigated molecular biology, mechanobiology, biomechanics, and tissue-based disease mechanisms associated with DDH during its development and progression to secondary hip osteoarthritis. The overall goal for this research was to comprehensively characterize these stages of DDH to elucidate mechanistic biomarkers for diagnosis, staging, and treatment monitoring as well as targets for novel prevention and treatment strategies.

The research focused on biomechanics of DDH utilized cadaveric hip specimens tested during physiologic range of motion movements to evaluate the stabilizing contributions of acetabular labrum and ligamentum teres. There was a large amount of variability among specimens, such that statistical analyses assumptions were violated, and caution must be taken when interpreting the results. However, the biomechanical testing data indicated that the acetabular labrum primarily provides stabilization in the lateral plane while the ligamentum

teres primarily provides stabilization in the anterior plane during hip movements of daily living. These results suggest that each of these soft tissue structures are likely overloaded in dysplastic hips such that their metabolic responses may characterize the development and progression of DDH in patients.

The research focused on mechanobiology included the analysis of dysplastic and non-dysplastic canine hip tissues cultured under physiologic and supraphysiologic tensile loads using bioreactor protocols designed to model relevant activities of daily living in a translational model. The results indicated that the ligamentum teres and acetabular labrum cells from dysplastic hips, and those subjected to supraphysiological tensile loading, regardless of hip status, upregulated inflammation-related biomarker production. The synovium did not increase production of measured biomarkers under supraphysiological tensile loading or in association with dysplasia. When the dysplastic labrum and ligamentum teres cells were placed under physiologic loading, their protein production profiles were similar to the corresponding healthy-hip tissues. These data corresponded well with the biomechanical testing data, supporting the hypothesis that labrum and ligamentum teres may be primary soft-tissue responders to supraphysiologic tensile loading in dysplastic hips. Importantly, physiologic loading of dysplastic ligamentum teres and labrum was associated with restoration of healthy-hip metabolic profiles, suggesting a correction of load on these tissues may reduce inflammatory mediators released to the joint and potentially mitigate progression to osteoarthritis.

The tissue-based research focused on characterization of metabolic responses by intra-articular hip tissues - ligamentum teres, acetabular labrum, synovium, and femoral head cartilage from individuals undergoing total hip arthroplasty for symptomatic hip osteoarthritis – in explant culture. The results showed that the ligamentum teres produced primarily degradative biomarkers, while the synovium produced a similar degradative profile as well as a number of inflammation-related biomarkers. The femoral head cartilage produced a biomarker profile that contained degradative, anabolic, and inflammatory factors. The metabolic responses of the labrum were more similar to cartilage than synovium and ligamentum teres. These results indicate that there may be tissue-specific mechanistic targets for optimally mitigating the dysplastic processes that lead to joint degeneration and progression to hip OA. Importantly, acetabular labrum metabolic profiles observed trends to histopathology assessments. The early stages of labral degeneration, indicated by lower histopathology severity scores, observed trends with increases in inflammatory and degradative biomarkers. The late stages of degeneration, indicated by higher histopathology severity scores, observed trends with an increase in anti-degradative biomarkers. These results lend further support to the support for further consideration of mechanistic biomarkers for use in screening, early diagnosis, and identification of therapeutic targets for DDH.

Clinical studies analyzing human serum and urine for biomarkers that may distinguish DDH, secondary hip OA, and healthy-hip status were performed. When analyzing concentrations of individual protein biomarkers, DDH prior to OA

progression was most associated with elevated levels of clinically relevant inflammatory and anabolic biomarkers analyzed, while DDH-related secondary hip OA was most associated with elevated levels of the biomarkers which are clinically relevant degradation-related. Further, DDH prior to OA progression was also associated with lower levels of bone metabolism and anti-degradative biomarkers. When protein biomarkers that were significantly different in the pre-OA DDH patients compared to their age cohort matched healthy-hip controls were grouped and analyzed as panels, the panel that combined two urine and two serum biomarkers had excellent discriminatory capability. These results suggest that panels of protein biomarkers measured in the urine and serum provide the potential for a non-invasive, cost-effective method for timely screening of DDH in this at-risk for developing secondary OA population. Further development and validation of these biomarker panels may result in highly sensitive and specific tools for early diagnosis, staging, and prognostication of DDH, as well as treatment decision making and monitoring capabilities filling a critical unmet need in healthcare.

Taken together, this body of work provides foundational data for characterizing the molecular biology, mechanobiology, biomechanics, and tissue-based disease mechanisms associated with DDH during its development and progression to secondary hip osteoarthritis. Candidate biomarkers for diagnosis, staging, and treatment monitoring, and targets for novel prevention and treatment strategies have been identified. To continue this translational research, next-step biomechanical studies could use the results of this work to develop and test finite

element and multibody modeling experiments that also include data from human subjects with healthy, dysplastic, and osteoarthritic hips. Mechanobiology and tissue-based experiments could be synergized to have more direct clinical relevance; co-culture and functional tissue studies in bioreactors using tissues from DDH patients would allow for the most valid clinical application. Finally, application of the biomarker panels to an infant population as well as prospective longitudinal testing in a larger and more diverse young adult population would provide the validation needed to implement this potential game-changing tool into clinical practice. Overall, this programmatic translational research provides a body of foundational work for progress towards evidence-based screening, early diagnosis, staging, and treatment decision-making and monitoring for DDH towards the ultimate goal of mitigating its symptoms and progression to secondary hip OA in the hundreds of thousands of individuals affected by this orthopaedic disorder.

VITA

Preston N. Wolfe was born in Columbia, Missouri, on May 4, 1994. He grew up in Ashland, Missouri and attended the Southern Boone School district and graduated from Southern Boone Country High School in May 2013. The following August he enrolled at William Woods University in Fulton, Missouri where he competed for four seasons in Track and Field. He graduated in May 2017 receiving the degree of Bachelor of Science in Biology with minors in Chemistry, Physics, and Physical Science. He entered University of Colorado-Anschutz in August 2017 in the Master of Science Modern Human Anatomy program. After one semester he transferred to the University of Missouri-Columbia and began graduate school in May 2018. He received a Doctor of Philosophy Degree in Pathobiology Area Program in August 2022.