SYNOVIAL FLUID LUBRICATION IN DISEASE: CHARACTERIZATION, MECHANISMS, AND THERAPY

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Cartilage exhibits remarkable low-friction and low-wear properties, which enable the tissue to withstand decades of repetitive loading cycles in vivo. Though osteoarthritis (OA) pathogenesis is highly variable across patients and multifactorial, inferior cartilage lubrication is a hallmark of OA. Despite the critical role of lubrication to joint health, our understanding of lubrication in disease is limited. Specifically, it is unclear how both of the critical synovial fluid lubricants, lubricin and hyaluronic acid (HA), are affected across different forms of OA. Furthermore, it is unclear how alterations in composition ultimately affect the lubricating properties of synovial fluid. Therefore, the overarching goal of this dissertation was to characterize synovial fluid lubrication in different forms and stages of OA. A holistic approach was applied whereby synovial fluid was examined for both lubricin and HA content as well as its mechanical properties including multi-modal friction coefficients, viscosities, and effective viscosities. This work contributes to the field's knowledge of lubrication in disease and advances several important and novel concepts; notably, (1) the Stribeck framework was applied successfully for the first time to synovial fluid, (2) effective viscosities of synovial fluid were calculated and applied to compare lubricating properties, and (3) finally, the lubricating effect of HA viscosupplementation, a common OA therapeutic, was found to depend on synovial fluid composition.

BIOGRAPHICAL SKETCH

Elizabeth Feeney attended the University of Pennsylvania and earned her Bachelor of Science in Engineering in 2014. As a Bioengineering major, she conducted tendon research for three years in the McKay Orthopaedic Research Laboratory under the mentorship of Dr. Louis J. Soslowsky and several graduate students, which resulted in two co-authored research articles and several conference abstracts. She also conducted research at the National Institutes of Health as part of the Bioengineering Summer Internship Program. She was awarded a Graduate Research Fellowship from the National Science Foundation before enrolling at Cornell University.

In 2014, she began her PhD in biomedical engineering at Cornell and joined the Bonassar Laboratory. During her research, she collaborated with other laboratories at Cornell including the Putnam, Reesink, and Paszek Labs. Over the course of her PhD work, she has co-authored two publications on lubricin mimetics and recombinant lubricin. She also has published in the Journal of Orthopaedic Research as first author. She has attended the Orthopaedic Research Society Annual Meetings, Osteoarthritis Research Society International, and the Summer Bioengineering Conference (SB3C). She earned two awards at these conferences for oral presentations.

For Ma, my best friend who went through the “valley of death” for me.

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LIST OF ABBREVIATIONS

Abbreviation/Symbol	**Description**
ACLT	Anterior cruciate ligament transection or tear
Ab	Antibody
a	Contact length
c	Mass concentration of polymer
ELISA	Enzyme-linked immunosorbent assay
μ	Friction coefficient
$F_{\parallel}$	Friction force
F_N	Normal force
HA	Hyaluronic acid
HYAL	Hyaluronidase
IA	Inflammatory arthritis
IA-Aberrant, IA-Typical	Inflammatory arthritis with either “typical” or “aberrant” tribological behavior
IHC	Immunohistochemistry
mRNA	Messenger RNA
mAb	Monoclonal antibody
NI	Non-inflammatory, as in NI osteoarthritis
n	Number of polymers per solution volume
OA	Osteoarthritis
PMN%	Polymorphonuclear neutrophil percentage
PNA	Peanut agglutinin
PCLT	Posterior cruciate ligament transection or tear
RA	Rheumatoid arthritis
RMS	Root Mean Square
RT-PCR	Reverse transcriptase–polymerase chain reaction

η_{sp}	Specific viscosity
S	Sommerfeld Number
SF	Synovial fluid
UDP	Uridine diphosphate
v	Sliding Speed
V	Volume of each polymer
η_0	Zero shear rate viscosity
$[\eta]$	Intrinsic viscosity
$\dot{\gamma}$	Shear rate
μ	Friction coefficient
μ_o, μ_{min}	Constant values of friction coefficient for equation fitting

CHAPTER 1

Cartilage Lubrication: Mechanisms, Players, and Disease

1.1 Abstract

Osteoarthritis (OA) is a degenerative, whole joint disease characterized by pain, pathological tissues changes, and loss-of-function. In health, synovial joints withstand high loads several times body weight for millions of loading cycles without wear. This remarkable ability derives from both the cartilage lining the ends of the bones and the synovial fluid that bathes the tissue. Though OA pathogenesis is highly variable across patients and multifactorial, inferior lubrication is a hallmark of OA. Herein, the properties and constituents of synovial fluid are discussed in the context of normal function and joint disease. The glycoprotein, lubricin, and hyaluronic acid (HA), are recognized as the key lubricants in synovial fluid. Additionally, background on lubrication, and particularly cartilage lubrication is reviewed. Finally, though synovial fluid alterations are well-recognized, holistic studies of the compositional and mechanical changes in disease are lacking. This gap in the literature underpins the motivation for studies discussed in subsequent chapters in this thesis, which seek to elucidate synovial fluid changes in different forms of OA.

1.2 Background on Osteoarthritis

Diarthrodial or synovial joints, such as the knee and shoulder joints, are highly mobile and comprised of several tissues enclosed within a synovial cavity. A thick, fibrous capsule surrounds the joint cavity, and its interior is lined by the synovium. The synovium produces synovial fluid, which is a viscous fluid bathing all tissues in the joint. This fluid lubricates tissues, including articular cartilage, and secondly, it acts as a medium for nutrient and waste exchange between

these tissues and the body. Opposing bones of the joint are lined by articular cartilage and connected by ligamentous tissue. Articular cartilage is hyaline cartilage, and it works to distribute load across the joint and minimize friction and wear during joint loading, including activities like running and walking. Ligaments join bones together and stabilize the joint. Together, these tissues enable low-friction movement for decades of life.

Osteoarthritis (OA) is a degenerative whole joint disease characterized by pain, loss-of-function, and pathological tissue changes. It is multifactorial in nature and manifests heterogeneously among patients, which has motivated the orthopedics community to recognize OA as a family of diseases. Numerous risk factors for OA are known including a history of joint trauma, genetics, aging, race, obesity, and sex [1]. The joint exhibits several tissue changes as well: cartilage loss, effusion, osteophytes, thickening of the subchondral bone, inflammation of the synovium, and ligamentous and meniscal degeneration. There are no currently approved therapies for modifying the progression of OA. Treatment initiates conservatively with use of non-steroidal anti-inflammatory drugs, physical therapy, and as needed, weight loss regimens. Further treatment options include intra-articular injection of hyaluronic acid viscosupplements and corticosteroids, which can provide temporary relief to a patient. Ultimately, many patients must resort to total joint replacement to overcome the pain and limitations of OA.

Effective lubrication is essential to a healthy joint, and inferior lubrication has been recognized as a hallmark of OA. Lubricating mechanisms become compromised by compositional and mechanical changes in synovial fluid and cartilage. Inferior lubrication results in higher tissue strains, which promotes cartilage wear [2,3] and chondrocyte death [4–6]. Chondrocytes are essential to maintaining the cartilage extracellular matrix and their death has been linked to matrix

degradation and the progression of OA [7]. To understand how lubrication becomes affected in OA, it is first necessary to consider the key synovial fluid lubricants, lubricin and hyaluronic acid.

1.2.1.A Lubricin

Lubricin is a glycoprotein produced by synoviocytes and chondrocytes that is well-established as a boundary lubricant of articular cartilage. Originally referred to as "lubricating glycoprotein-1," it was renamed lubricin by Swann et al. in 1981 in an investigation of its size and molecular weight [8]. This name is frequently interchanged with the name of the gene encoding lubricin, proteoglycan 4 or PRG4. Lubricin is homologous to other mucinous proteins including superficial zone protein (SZP), megakaryocyte-stimulating factor precursor (MSF), and camptodactyly-arthropathy–coxa vara–pericarditis (CACP) protein [9].

Lubricin resembles a bottlebrush in structure, and it consists of a highly glycosylated core flanked by N- and C-termini. It is 1,404 amino acids long, approximately 200nm in contour length [8,10], and approximately 228kDa in weight depending on its glycosylation [9]. Of particular interest for its lubricating properties is the mucin domain. This domain consists of imperfect repeats of the amino acid sequence KEPAPTT, which are extensively glycosylated with O-linked β(1–3)Gal-GalNAc oligosaccharides partially capped with sialic acid residues [9,11]. This region is consequently negatively charged and hydrophilic, which enables lubricin to attract water and form a hydrated, lubricating layer at the cartilage surface [12–14]. Furthermore, interaction between surfaces is limited by steric repulsion between brushes leading them to compress within themselves rather than interpenetrate with brushes on the opposite surface [12,13,15,16]. Enzymatic digestion of sialic acid and oligosaccharide groups of lubricin were shown to reduce lubricating ability by 19% and 77%, respectively [17]. Additionally, binding of lubricin is essential for effective boundary lubrication [14]. At the N-terminus are two somatomedin domains and a

hemopexin-like domain at the C-terminus. The N-terminus is thought to facilitate aggregation among lubricin molecules while the C-terminus is thought to aid in binding of lubricin to cartilage [18].

Early studies of lubrication recognized mucin as a critical component of synovial fluid. After the formal discovery of lubricin, Swann demonstrated that lubricin reduces cartilage-on-glass friction coefficients in a concentration-dependent manner [19]. Numerous studies using various tribometer types and surface materials have validated this finding [20–24].

Lubricin is most recognized for its potent lubricating capacity and role in arthritis. Precocious joint failure is a hallmark of camptodactyly-arthropathy–coxa vara–pericarditis (CACP) syndrome, which arises from a mutation in the PRG4 gene [25]. Lubricin-knockout mice were developed and shown to recapitulate CACP syndrome well [25]. Excluding CACP, the earliest investigation into lubricin's role in arthritis was conducted by Elsaid et al. in 2005 in an animal model of post-traumatic osteoarthritis induced by transection of the anterior and posterior cruciate ligaments of the knee in rabbits [2]. This study demonstrated a correlation between the lubricating properties of synovial fluid, lubricin concentrations, and matrix degradation. Subsequent studies have found that lubricin decreases in several arthropathies [26–30] and that treatment of the joint with exogenous lubricin can prevent OA progression [31–37]. However, other studies have found lubricin to increase in disease [38–45] or exhibit no change [46]. At present, the role of lubricin in OA is poorly understood.

1.2.1.B Hyaluronic Acid

Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan that confers synovial fluid with its viscoelastic properties. HA is a disaccharide of D-glucuronic acid and *N*-acetyl D-glucosamine, which exists in linear chains ranging from kilodaltons to megadaltons in size. It is produced by

HA synthases on the cell surface of type B synoviocytes in the synovium, and to a lesser degree, chondrocytes [60,61]. At physiological pH, the carboxyl groups of glucuronic acid are negatively charged (pK_a=3.2 [47]), thus conferring HA polyanionic. HA has been a focus of arthritis research for decades because of the profound loss of synovial HA, shift toward low molecular weight forms of HA in synovial fluid, decreased synovial fluid viscosity, and application of HA viscosupplements in diseases like osteoarthritis and rheumatoid arthritis. Recent studies have also highlighted the biological effects of HA on synovial joint tissues, including its anti-inflammatory properties and ability to stimulate endogenous HA production in vitro [48].

Synovial fluid's viscous and elastic properties are modulated by the concentration and size of HA. HA forms wormlike random coils that can be approximated as a sphere with a volume that scales with the molecular weight raised to the 1.8 power [49]. Given that synovial fluid contains HA on the order of megadaltons in size, the molecules occupy large volumes.

For sphere-like HA in physiologic saline, the intrinsic viscosity, [η], varies as a function of its molecular weight as described by the Mark–Houwink–Sakurada equation [49]:

$$[\eta] \sim M^{0.8} \tag{1}$$

The specific viscosity of dilute HA solutions can be predicted using equation (2) by the volume fraction of polymer in solution and the volume of each polymer:

$$\eta_{sp} = c[\eta] = 2.5nV \tag{2}$$

where η_{sp} is the specific viscosity, c is the mass concentration of the polymer, n is the number of polymers per solution volume, and V is the volume of the polymers. As HA solutions become less dilute, the random coils of HA interact and interpenetrate with one another, which leads to a dramatic increase in the solution viscosity that cannot be described by Equation (1). The concentration at which this coil overlap occurs is inversely proportional to the molecular weight

of the HA. The non-ideality has been modeled and refined instead using a series exponential approximation of viscosity as a function of HA molecular weight and concentration [49].

Synovial fluid exhibits shear-thinning and viscoelastic properties resulting from its HA content. Under shear, HA molecules can deform and stretch in the direction of flow. Aligned and stretched polymers do not contribute as strongly to viscosity as coiled polymers. At low shear rates, HA molecules can relax to their undeformed state, but as the shear rate is increased, fewer molecules are able to relax and the viscosity of the solution decreases. The magnitude of shear thinning and the relaxation time are proportional to the molecular weight and concentration of HA. Under rapid cyclic shearing, HA polymers behave elastically because they cannot deform and flow at the same time scale as the applied deformation.

1.3 Lubricating Mechanisms of Cartilage

Cartilage exhibits remarkable low-friction and low-wear properties, which enable the tissue to withstand decades of repetitive loading cycles in vivo. Friction is the contact force between surfaces that opposes their relative motion against one another. The friction coefficient, μ, is a dimensionless term that quantifies the degree of resistance between opposing surfaces calculated as the ratio of the tangential friction force over the normal force:

$$\mu = \frac{F_{\parallel}}{F_N} \tag{1}$$

Contact pressures across the cartilage surface are nonuniformly distributed and have been measured as high as 18MPa in the hip [50,51]. Typical loads during walking range from 1.2-7.8 times body weight in the knee and 2.5-6 times body weight in the hip [50,52]. Relative cartilage sliding speeds are somewhat debated in the field and have been published as 0-0.3mm/s [51], <1mm/s [53], 0.5-5mm/s [54], ~100mm/s [55], and up to 300mm/s [56]. Friction coefficients of cartilage have been measured to be on the order of 0.001 or less [57–59], which is approximately

five times lower than Teflon [53]. Cartilage has a small pore network and charged proteoglycan matrix that enables normal loads to be supported by water trapped inside the matrix as opposed to the solid tissue component, a mechanism known as interstitial fluid pressurization which contributes to cartilage's extremely low friction coefficients [54]. Several theories to explain cartilage lubrication have been proposed and debated since the 1960s, though here, only boundary mode lubrication, mixed mode lubrication, and fluid-film lubrication will be discussed. Theories including interstitial fluid pressurization, squeeze-film, boosted lubrication, and weeping lubrication have been well-reviewed elsewhere [56,60]. While there are many cartilage lubrication mechanisms operating in concert, the Stribeck framework has been successfully applied to describe the magnitude of cartilage friction across different loading regimes occurring in a joint [22,39,61–64]. Lubricin and HA are critical to lubrication across these modes.

1.3.1 Stribeck Framework for Analyzing Lubrication

Synovial joints are lubricated by two fundamental mechanisms during daily movement: boundary lubrication and fluid-film lubrication. Factors including the sliding speed, fluid viscosity, normal load, and contact area govern which lubrication mechanisms are occurring in the joint, which can lead to dramatic differences in cartilage friction. The Stribeck framework, originally developed to describe friction in journal bearings, is a system that captures how friction varies as a function of these factors (Figure 1-1). The Sommerfeld number is a dimensionless term that is calculated as the $\frac{\eta \nu a}{F_N}$ where η is the fluid viscosity, ν is the sliding speed, a is the contact width between surfaces, and F_N is the normal load.

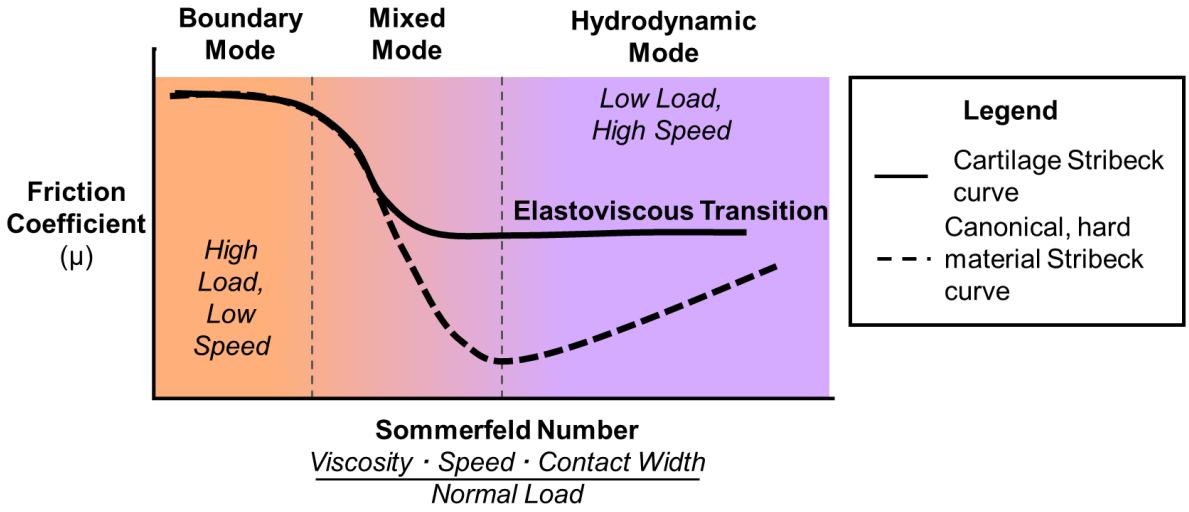

Figure 1-1: The Stribeck curve shows friction coefficient as a function of the dimensionless term, Sommerfeld number, which accounts for the fluid viscosity, normal load, sliding speed, and contact area of a system. Here, two Stribeck curves are shown: one of a hard material and the other showing cartilage.When friction is plotted as a function of Sommerfeld numbers, three modes of lubrication become evident: boundary mode, mixed mode, and hydrodynamic mode. The transition between these modes is governed by the magnitude of the Sommerfeld number. For example, increased viscosity or sliding speed, will move a system toward the low-friction, hydrodynamic mode. This framework has been applied to describe cartilage lubrication [22,39,61–64]. However, it is important to note that cartilage is very different from the journal bearings for which the Stribeck framework was developed; unlike metals in bearings, cartilage is soft, porous, and hydrated and lubricated by synovial fluid, a complex, biological fluid with multiple molecular components.

Boundary lubrication occurs when opposing joint surfaces are in almost complete contact but separated and protected by a layer of adsorbed molecules and proteins, so-called boundary lubricants (Figure 1-2). Friction results from the contact between opposing cartilage asperities. The boundary mode occurs in conditions of high load and slow sliding speeds such as during the strike phase of gait, and it is characterized by high friction coefficients (Figure 1-1). Lubricin is recognized as the predominant boundary lubricant of articular cartilage, though recent studies suggest hyaluronic acid may also function as a boundary lubricant.

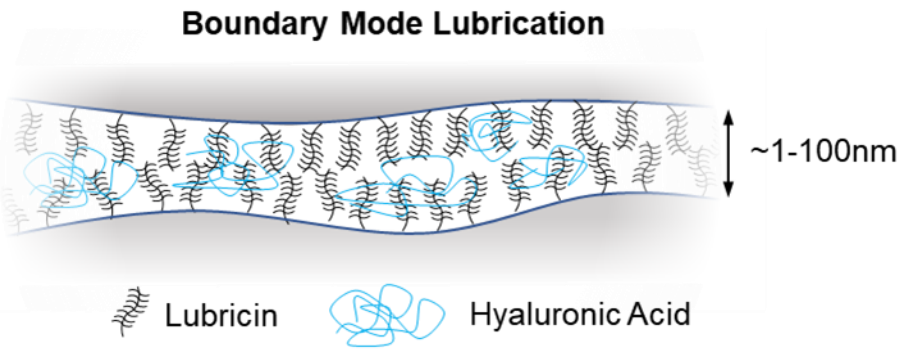

Figure 1-2: Cartoon illustrating boundary lubrication of articular cartilage. Lubricin adsorbs to and protects the cartilage surface. Hyaluronic acid is entangled with the lubricin and may also contribute to boundary lubrication.

With increasing Sommerfeld number, friction coefficient decreases as fluid-film lubrication mechanisms begin to dominate. The mixed lubrication mode is the transition mode between boundary mode lubrication and fluid-film lubrication, and it is the combination of these two lubrication mechanisms (Figure 1-1). In the mixed mode, viscous effects of the fluid result in partial separation of cartilage surface where the fluid film thickness is slightly greater than the asperities. Friction results from predominantly boundary-type contact between cartilage asperities that are not separated by a partial film [65]. From a classical engineering perspective, fluid-film lubrication occurs under low load and high sliding speed conditions such as during the swing phase

of gait (Figure 1-1), which allows for the formation of a fluid film that separates articulating surfaces and asperities to a greater degree than boundary lubrication. Fluid-film lubrication leads to extremely low friction coefficients because the load is supported by the fluid film. The friction depends on the shear forces in the fluid, which can become substantial at high Sommerfeld numbers and lead to a rise in friction coefficient (Figure 1-1). For cartilage and synovial fluid, HA is thought to facilitate the transition from boundary mode to the mixed and elastoviscous modes (see Section 1.3.2). HA's viscous nature confers it with an enhanced ability to pressurize and support fluid film formation compared to a non-viscous fluid.

As alluded to previously, there are distinct differences between cartilage and traditional bearings that affect lubricating mechanisms. The precise mechanisms by which cartilage is lubricated remain highly debated, including most notably, the ability for cartilage to sustain full fluid-films [22,54,63,66]. Friction coefficients of cartilage have been observed to decrease with increased sliding speed and reduced load, but they do not reach the minimal values obtained in full hydrodynamic lubrication of hard materials [22]. As opposing cartilage surfaces glide past one another, fluid is drawn between them and becomes pressurized. Unlike a hard, non-porous material, the fluid can escape into the porous network of cartilage and deform the soft cartilage. This phenomenon has been described as elastoviscous lubrication mainly in the context of hydrogels and more recently, cartilage [22,39,67]. The elastoviscous regime describes the region in the Stribeck curve in which cartilage friction coefficients plateau to a minimum friction coefficient greater than the minima of hard materials (Figure 1-1). Burris and Moore have even argued that the ability of cartilage to "tribologically rehydrate" is another mechanism of cartilage lubrication in which the imbibement of fluid into the cartilage reduces the normal load supported by the solid matrix thus leading to lower friction [68].

1.3.2 Lubricin, HA, and Lubricin-HA Synergy

Individually, lubricin and HA each contribute to lower friction coefficients. Lubricin acts as a boundary lubricant by binding to the cartilage surface and forming a hydrated layer of protection [12–14]. Dose-response analysis of boundary mode cartilage friction coefficients indicate that lubricin can decrease friction coefficients by as much as 70% compared to saline [19–21,69]. Friction coefficients have been found to decrease with increasing HA molecular weight and concentration in whole joints [70,71] and cartilage-cartilage tribometers [24,41,71–73]. The mechanisms by which HA lubricates cartilage is contentious specifically regarding whether it can act as a boundary lubricant. Studies that report decreased boundary mode friction coefficients with HA have not conducted Stribeck analysis to confirm measurements were conducted in the boundary mode [69,72,73], and the rotational tribometers employed may not facilitate development of fluid-film-like lubrication. HA lubrication measured via surface force apparatus with mica surfaces indicates HA must be chemically bound to the surface to function as a boundary lubricant [74–77]. An alternative theory proposes that HA lubricates cartilage by interacting with lubricin and shifting lubrication towards the elastoviscous mode per the theory of viscous boundary lubrication discussed below [22,71].

Lubricin and HA act synergistically to lubricate cartilage. Friction coefficients are lower in combined solutions of lubricin and HA than when they are measured independently (cartilage-cartilage rotational tribometers [24,69,72,78], latex-glass rotational tribometer [79], cartilage-on-glass linear tribometers [22] and other tribological systems [80]). Similar results have been observed for friction coefficients measured via surface force apparatus with chemically-grafted HA to mica [77] and atomic force microscopy with grafted HA and collagen type II [81]. Molecular-scale studies suggest the formation of a viscous gel layer consisting of lubricin-HA

complexes [77,81–83]. This layer contributes to viscous boundary lubrication [84], whereby there is an enhanced surface viscosity resulting from formation of the gel layer, which enables an earlier transition to the mixed and elastoviscous modes. This concept was supported by work by Bonnevie et al. demonstrating that lubricin tethered HA to the surface and facilitated a transition towards the elastoviscous lubrication mode at lower Sommerfeld numbers than HA alone [22].

The physical structure of a lubricin-HA complex and how it contributes to friction across the span of the Stribeck curve is unknown. It is also unclear if other lubricating molecules e.g., phospholipids contribute to this complex and lubrication. Current theories suggest that HA and lubricin form molecular entanglements though physical crosslinks that are electrostatic or mechanical in origin [22,72,77,82,85,86]. Lubricin's somatomedin-B-like and hemopexin-like domains are positively charged, and thus may interact with the negatively charged regions of HA. Hydrophobic interactions may also occurs between the nonpolar regions of HA and N- and C-termini of lubricin [77,87].

1.3.3 Effective Lubricating Viscosities

Synovial fluid viscosity has long been characterized using standard rheology techniques, which may not accurately capture viscous behavior at the cartilage surface. One limitation of standard rheology is that rotational rheometers require a fixed and constant gap between moving surfaces, which is usually on the order of hundreds of microns. This thickness is much larger than the estimated gap or film thickness of 100nm [88], if any, between moving cartilage interfaces. Furthermore, operating conditions of the joint (load, sliding speed) would modulate the gap distance, unlike the situation in a rheometer. In the joint, the effective shear rate is unknown, which is problematic since synovial fluid is shear-thinning. Consequently, the viscosities throughout the modes of the Stribeck curve cannot be accurately estimated or measured at present. Viscosity

measurements are also sensitive to artifacts resulting from the test setup; interfacial effects at the liquid-air interface and protein deposition on rheometer surfaces also contribute to measurement error [89]. Similarly, recent studies suggest molecular entanglement between lubricin and HA at the cartilage surface can result in local viscosities being much higher than bulk solution viscosities [22,84]. These limitations in studying viscous lubrication represent a major barrier and opportunity in the field of cartilage lubrication.

Viscous lubrication by shear-thinning materials may be better understood using the concept of "effective viscosity" instead of measured viscosities. The effective viscosity represents the fluid viscosity between surfaces that is calculated by collapsing measurements of friction versus sliding speed for a given lubricant onto a master Stribeck curve, and it has been applied to study tribology in steel-rubber and recently cartilage-glass interfaces [90–92]. In a study of the shear-thinning polymer, guar gum, in solution between a steel-rubber contact, it was found that collapsing data in this way better predicted and characterized fluid-film lubrication than using the zero-shear rate viscosity [90]. Increasing concentrations of guar led to an increase in effective viscosity and an earlier transition towards mixed and hydrodynamic lubrication modes, i.e., a leftward shift of the Stribeck curves. Bonnevie et al. observed a similar result while characterizing viscosupplement products; effective viscosities correlated with both measured friction coefficients and patient-reported pain scores following viscosupplement therapy better than measured rheological parameters like the zero-shear rate viscosity or viscoelastic moduli [91]. Calculation of the effective viscosity represents a useful tool for studying and comparing the fluids' lubricating properties.

1.4 Lubrication in Disease

Effective lubrication is essential to a healthy joint, and alterations in lubrication can contribute to the progression of osteoarthritis. Synovial lubricin and HA composition has been found to vary dramatically in OA as a function of OA type, severity, and time after injury in post-traumatic osteoarthritis. Most studies have examined changes to lubricin or HA alone and few have linked changes to synovial fluid mechanics. A review of the current literature on the changes in lubricin, HA, and synovial fluid friction is presented in the following sections: 1.4.1 and 1.4.2.

1.4.1 Hyaluronic Acid in Disease

Arthritic synovial fluid is often observed to exhibit decreased viscosity resulting from a loss of hyaluronic acid, especially high molecular weight forms. Investigations of the mechanical properties and HA content of pathologic and healthy synovial fluids were first conducted by Balazs and colleagues in the 1950s and 1960s [93,94], and have been well-reviewed by Fam et al. [95]. Healthy synovial fluids exhibit HA concentrations ranging from 1-4mg/ml [44,94,96–99]. Traumatic joint injuries and arthritis result in a decrease in HA concentrations to <1mg/ml and a shift towards low molecular weight polymers [38,39,41,44,98,100–103]. It is worth noting that concentration and molecular weight averages are quite variable and could be influenced by patient-to-patient variability, inclusion criteria, disease severity, and quantification assay.

Studies of synovial fluid mechanics and HA have largely focused on the rheological properties of synovial fluid including their steady shear properties (e.g., zero-shear rate viscosity, η_0, and the shear rate at which shear thinning begins) as well as viscoelastic properties (e.g., storage and loss moduli). In OA and rheumatoid arthritis fluids, zero-shear rate viscosities decreased by 2-3 orders of magnitude and the onset of shear thinning increased by 1-2 orders of magnitude compared to healthy control fluids. Likewise in disease, storage and loss moduli were an order of

magnitude lower and exhibited viscous behavior across a wider range of shear rates versus healthy fluids [104].

To counteract the loss of HA and viscoelastic properties of synovial fluid in OA, viscosupplementation therapy was developed. Viscosupplementation is the injection of mid- to high molecular weight HA into the joint for treating mild-to-moderate OA. Viscosupplements are a popular treatment, which have been used clinically for decades. There are several viscosupplements currently approved in the United States for treating knee OA, which vary according to their source (avian/non-avian), structure (cross-linked or non-cross-linked), concentration and volume, and dosing strategy (single vs. multiple injections). They result in reduced pain and increased range-of-motion, possibly due to biological effects of HA rather than through purely mechanical means [48]. However, clinical results of viscosupplementation are highly variable among patients [105,106], which has led major orthopedic associations to offer conflicting guidelines to clinicians on their use [106–109]. Their effectiveness could be modulated by factors such as age, effusion, and disease severity for example. Altogether, HA viscosupplements' mechanism of action and appropriate use criteria remain unclear.

1.4.2 Lubricin in Disease

Using various methodologies, lubricin has been found to both increase and decrease in arthritis and models of arthritis (**Error! Reference source not found.**). Decreases in lubricin have been found in antigen-induced arthritis in rats [26], humans patients [27], guinea pigs [28], and rats [29] with anterior cruciate ligament tears, and late stage osteoarthritis and rheumatoid arthritis in human patients [30]. Subsequent studies demonstrated the therapeutic benefits of intra-articular lubricin injections in several arthropathies including rat meniscal tear osteoarthritis model [33], rat anterior cruciate ligament tear model [32,34,36,37], and ovariectomized rats simulating

osteoarthritis in relation to post-menopausal osteoporosis [35]. In contrast, lubricin has been found to increase in several equine studies (cartilage impact injuries [38], osteochondral fragmentation [38–41], and full-thickness defects [38]), cranial cruciate ligament tears in canines [110], anterior cruciate ligaments tears in sheep [42], advanced human osteoarthritis [43], human articular fractures [44], and end-stage human osteoarthritis [45]. One study of human patients with symptomatic osteoarthritis found no difference in synovial fluid concentrations of lubricin [46]. In these cases where lubricin is not lowered in disease, the therapeutic benefit of exogenous lubricin is unknown.

The relationship between lubricant content and friction in disease is poorly understood. Both cartilage friction coefficients and synovial fluid friction coefficients have been reported to increase in disease (**Error! Reference source not found.**). Importantly, few studies measure both lubricin content in synovial fluid and friction, including only 9 of the 16 in **Error! Reference source not found.**. Decreased lubricin concentrations and expression have been associated with increased friction coefficients [2,3,26,28,46], but studies examined different arthritis models, species, and tissues (cartilage or synovial fluid) and use different types of tribometers. In other studies that observed lubricin to increase in disease, friction coefficients of synovial fluid [41,42,44] and cartilage [43] were increased compared to controls or showed no change [111]. Inferior friction was instead linked to a loss of HA [41,42,44].

Table 1-1: Measurements of lubricin and friction in various arthropathies and animal models

Citation	Effect of Disease on Lubricin	Effect of Disease on Friction	Species	Injury/ Model/ Disease	Tribometer Type, Tissue Tested for Friction	Lubricin Control Group Used	Friction Control Group	Tissue	Lubricin Quantification method
Elsaid, K. A. et al., 2005, Arthritis Rheum., **52**(6). [2]	Decreased concentration at 2- and 3-weeks post-injury relative to week 1	Increased boundary friction at 2- and 3-weeks post-injury relative to week 1.	Rabbit	ACLT & PCLT	Latex glass rotational tribometer, SF	Week 1 post-injury concentration	Saline	SF	Sandwich ELISA (Capture Reagent: PNA, Detection Reagent: amino-terminus-specific Ab (J108), Standard: human lubricin purified from SF)
Elsaid, K. A. et al., 2007,Arthritis Rheum., **56**(1). [26]	Decreased expression at days 4 and 7 post-induction relative to control	Increased boundary friction	Rat	Antigen-induced arthritis	Cartilage pendulum tribometer, cartilage	Contralateral control limb	Contralateral control limb	Synovial tissue	Quantitative RT-PCR
Elsaid, K. A. et al., 2008, Arthritis Rheum., **58**(6). [27]	Decreased concentration after ACLT	N/A	Human	ACLT, naturally occurring		Contralateral control limb		SF	ELISA-c
Teeple, E. et al., 2008, J Orthop. Res., **26**(2). [28]	Decreased concentrations relative to (A) contralateral control 9 months post-ACLT, (B) 3-month old healthy control, (C) 12-month old guinea pigs	Increased friction coefficient in ACL-deficient knees compared to contralateral control knees, knees with mild OA, and knees with no OA	Hartley guinea pig	ACLT	Cartilage-cartilage pendulum tribometer, cartilage	Contralateral control, 3-month old control (no OA), 12-month old (mild OA)	Contralateral control, 3-month old control (no OA), 12-month old (mild OA)	SF	ELISA-c
Elsaid, K. A. et al., 2009, Arthritis Rheum., **60**(10). [29]	(1) Decreased concentrations at weeks 1 and 4 post-ACLT, (2) Decreases expression of lubricin relative to contralateral control, (3) Reduced lubricin staining on cartilage surface	N/A	Rat	ACLT		Contralateral control		(1) SF, (2) Synovial membrane, (3) Cartilage	(1) ELISA-a, (2) RT-PCR, (3) 9G3-based IHC

Table 1-1: Measurements of lubricin and friction in various arthropathies and animal models

Citation	Effect of Disease on Lubricin	Effect of Disease on Friction	Species	Injury/ Model/ Disease	Tribometer Type, Tissue Tested for Friction	Lubricin Control Group Used	Friction Control Group	Tissue	Lubricin Quantification method
Kosinska, M. K. et al., 2015, PLoS One, **10**(5). [30]	Decreased in late OA and RA relative to control	N/A	Human	RA and OA		Post-mortem SF		SF	ELISA-b
Ludwig, T. E. et al., 2012, Arthritis Rheum., **64**(12). [46]	Variable concentrations; similar concentrations between control fluids and OA fluids	PRG4-deficient SF exhibited higher kinetic friction coefficients than normal SF	Human	Symptomatic knee OA	Cartilage-cartilage rotational tribometer, SF	Normal post-mortem SF	Normal post-mortem SF, saline	SF	ELISA-b
Wanderling, C. et al., 2018, Clin. Appl. Thromb. Hemost., 24(1). [45]	Increased concentrations relative to control	N/A	Human	End-stage OA		Healthy patients' plasma		Blood	Plasma ELISA, MyBioSource.com
Peal, B. T. et al., 2020, J. Orthop. Res. [38]	Increase in lubricin all injury models relative to controls	N/A	Horse	Carpal osteochondral fragmentation, talar partial-thickness cartilage impact injury, femoral lateral trochlear ridge full-thickness cartilage defects		Day 0 SF, i.e., baseline SF prior to injuries		SF	ELISA-a
Wang, Y. et al., 2019, Osteoarthr. Cartil., **27.** [110]	Increased concentrations	N/A	Canine	Cruciate ligament tear		Healthy canines		SF	Sandwich ELISA

Table 1-1: Measurements of lubricin and friction in various arthropathies and animal models

Citation	Effect of Disease on Lubricin	Effect of Disease on Friction	Species	Injury/ Model/ Disease	Tribometer Type, Tissue Tested for Friction	Lubricin Control Group Used	Friction Control Group	Tissue	Lubricin Quantification method
Feeney, E. et al., 2019, J. Orthop. Res., **37**(5). [39]	Increased concentrations	No change in boundary or eleastoviscous mode friction coefficients. Reduced transition number after injury.	Horse	Osteochondral fracture	Cartilage glass tribometer, SF	Day 0/baseline SF prior to injuries	Day 0/baseline SF prior to injuries	SF	ELISA-a
Reesink, H. L. et al., 2017, Osteoarthr. Cartil., **25**(1). [40]	(1) Increased concentrations, (2) Increased gene expression in synovial membrane but not cartilage relative to controls, (3) Increased lubricin immunostaining in synovium relative to contralateral tissue	N/A	Horse	Osteochondral fracture (naturally occurring and induced fracture)		Contralateral control limb		(1) SF (2,3) Synovial membrane, (3) Osteochondral section	(1) ELISA-a (2) RT-PCR, (3) IHC (mAb 6A8)
Antonacci, J. M. et al., 2012, Arthritis Rheum., **64**(9). [41]	Increased concentrations	Increased boundary friction in acute injury SF compared to normal SF.	Horse	Osteochondral fracture (acute and chronic)	Cartilage-cartilage rotational tribometer, SF	Normal SF from contralateral control joints	Normal SF from contralateral control joints	SF	ELISA with mAb GW4.23 and purified equine lubricin as the standard
Neu, C. P. et al., 2010, Arthritis Rheum., **62**(9). [43]	(1) Increased concentrations	Increased friction coefficient correlated with OA score	Human	End-stage OA	Cartilage-glass tribometer, cartilage	Post-mortem tissue	Post-mortem tissue	(1) SF (2) Osteochondral sections, (3) Cartilage	(1,3) ELISA-c (2) IHC

Table 1-1: Measurements of lubricin and friction in various arthropathies and animal models

Citation	Effect of Disease on Lubricin	Effect of Disease on Friction	Species	Injury/ Model/ Disease	Tribometer Type, Tissue Tested for Friction	Lubricin Control Group Used	Friction Control Group	Tissue	Lubricin Quantification method
Ballard, B. L. et al., 2012, J. Bone Jt. Surgery-American Vol., **94**(10).	Increased concentrations	Increased boundary mode friction coefficients relative to control fluid	Human	Osteochondral fracture	Cartilage-cartilage rotational tribometer, SF	Normal SF from contralateral control joints	Normal SF from contralateral control joints	SF	Western blot
Atarod, M., et al., Osteoarthr. Cartil., 23(4).	Increased concentrations at 2 and 4 weeks post-ACLT relative to 20 weeks	Increased SF friction coefficients at 2- and 4-weeks postt-injury relative to 20 weeks	Sheep	Anterior cruciate ligament tear	Cartilage-cartilage rotational tribometer, SF	N/A	Healthy bovine SF, PBS	SF	ELISA-b

Table 1-1 Legend: Yellow shading denotes studies that observed decreased lubricin, while blue denotes studies that observed increased lubricin. IHC≡Immunohistochemistry, Ab≡antibody, SF=synovial fluid, N/A≡Not measured, ELISA-a≡Sandwich ELISA (Capture Reagent: PNA, Detection Reagent: 9G3 Ab Standard: Lubricin purified from SF), ELISA-b≡Sandwich ELISA (Capture Reagent: Ab against lubricin's C-terminus (LPN), Detection Reagent: PNA conjugated with horseradish peroxidase, Standard: lubricin purified from culture media), ELISA-c≡ Sandwich ELISA (Capture Reagent: PNA, Detection Reagent: mAb S6.79 or S6.89, Standard: Superficial zone protein purified from cultured explants' media

1.4.3 Open Questions in Lubrication

While lubrication is critical to joint health, lubrication in OA is poorly understood. Lubrication's variability is also apparent, which adds to a growing recognition that OA is a family of diseases with similar end-stage outcomes but varying pathogenesis. For example, lubricin and HA content in synovial fluid varies significantly with injury type in PTOA, with time, and disease severity. Despite the wide variation in lubricin and HA in disease and importance of lubrication to joint health, lubricin-HA interactions have been measured for only a narrow subset of lubricin and HA conditions. Few studies have quantified both the composition and frictional properties of arthritic synovial fluid; of these, some have focused only on lubricin [2,28,43] and other more recent reports have examined both lubricin and HA [41,42,44,111]. Given the synergy between lubricin and HA, it is critical that lubrication be examined holistically.

Furthermore, in arthritic synovial fluid and modeled conditions, cartilage lubrication has not been examined across all physiologic loading conditions or regimes of the Stribeck curve. This is a critical knowledge gap because there are several mechanisms of cartilage lubrication at play during gait that govern cartilage friction coefficients. Currently, Stribeck analysis has not been adopted across the field. Consequently, with research groups using different tribometers and operating conditions in their studies, it is challenging to make comparisons among studies and synthesize results. Because increased friction can predispose cartilage to degradation in OA [2,3], it is important to understand how lubricant composition affects cartilage friction coefficients across all modes of lubrication.

Finally, this line of research has important consequences on the development and application of lubrication-based therapies for treating OA. These include HA viscosupplements (Section 1.4.1) and other pre-clinical therapies like lubricin tribosupplements [31–33] and

lubricant mimetics [112,113]. Lubricin and HA differentially affect friction coefficients across lubrication modes. By examining multi-modal lubrication changes, we can better understand how lubrication contributes to OA pathogenesis and inform clinical interventions concerning the type of lubricant therapy to administer.

Therefore, the goal of the research discussed in the subsequent chapters is to examine lubrication holistically in different forms and stages of OA by application of the Stribeck framework. The overarching hypothesis was that:

Hypothesis: The Stribeck framework and effective viscosity are useful tools to describe cartilage lubrication by synovial fluid and HA viscosupplements.

The specific goals in each chapter were as follows:

Specific Aim 1: To examine temporal changes in lubricin, HA, and synovial fluid lubrication over time in the equine carpal osteochondral fragment model of PTOA.

Specific Aim 2:

(A) To investigate the differences in the lubricating properties of synovial fluid from two different populations of patients with either non-inflammatory OA or inflammatory arthritis.

(B) To investigate how supplementation with the modified HA viscosupplement, Hymovis®, affects lubrication in synovial fluids derived from non-inflammatory OA or inflammatory arthritis patients.

Specific Aim 3:

(A) To quantify the compositional and mechanical changes in synovial fluid following anterior cruciate ligament tear injury and reconstruction

(B) To determine the effect of the modified HA viscosupplement, Hymovis®, on synovial fluid lubrication after anterior cruciate ligament tear injury and reconstruction in vitro

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CHAPTER 2 [1]

Temporal Changes in Synovial Fluid Composition & Elastoviscous Lubrication in the Equine Carpal Fracture Model

2.1 Abstract

The objective of this study was to examine temporal variations in synovial fluid composition and lubrication following articular fracture. Post-traumatic osteoarthritis (PTOA) was induced by creating an osteochondral fracture in the middle carpal joint of four horses while the contralateral limb served as a sham-operated control. Horses were exercised on a high-speed treadmill, and synovial fluid was collected pre-operatively and at serial timepoints until 70 days post-operatively. Lubricin and hyaluronic acid (HA) concentrations were measured using sandwich ELISAs, and the molecular weight distribution of HA was analyzed via gel electrophoresis. Synovial fluid viscosity and cartilage friction coefficients across all modes of lubrication were measured on days 0, 19, 33, and 61 using a commercial rheometer and a custom tribometer, respectively. HA concentrations were significantly decreased post-operatively, and high molecular weight HA (>6.1MDa) did not recover to pre-operative values by the study termination at day 75. Lubricin concentrations increased after surgery to a greater extent in the OA as compared to sham-operated limbs. Viscosity was significantly reduced after surgery. While boundary and elastoviscous mode friction coefficients did not vary, the transition number, representing the shift between these modes, was lower. Although more pronounced in the OA

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limbs, similar derangements in HA, HA molecular weight distribution, viscosity and transition number were observed in the sham-operated limbs, which may be explained by synovial fluid washout during arthroscopy.

2.2 Introduction

Post-traumatic osteoarthritis (PTOA) accounts for an estimated 12% of symptomatic OA in the lower extremities of adults in the United States [1]. In particular, articular fractures have been estimated to increase the risk of PTOA by more than twenty-fold [2]. The standard-of-care for treating articular fractures by surgically restoring surface congruity and anatomic alignment fails to prevent the progression of OA [3]. Traumatic loading has been shown to initiate a cascade of inflammatory events that contribute to the pathogenesis of PTOA and cartilage breakdown via chondrocyte apoptosis and extracellular matrix degradation [4,5]. Furthermore, cartilage wear may be exacerbated by a loss of protective cartilage lubricants [6] that are downregulated by or made more vulnerable to enzymatic degradation by pro-inflammatory cytokines like TNF-α and IL-1β [7–9]. Traumatic joint injuries pose an identifiable risk factor for PTOA, and growing evidence suggests that interventions which are targeted at restoring joint homeostasis and applied acutely after injury may prevent OA development [2].

The lubricating capacity and composition of synovial fluid and its lubricant composition have been found to vary widely after traumatic injury. Poor lubrication has been associated with cartilage damage and may play a key role in OA progression both through direct mechanical wear and induction of cell death and apoptosis due to mechanical overloading [6,10,11]. Synovial fluid contains two critical cartilage lubricants: lubricin and hyaluronic acid (HA). HA, a linear polymer that imparts synovial fluid with its viscous properties, has been found to decrease in human patients with clinical OA progression and in many animal models of PTOA [12–19]. Decreased

concentrations and expression of lubricin, a mucinous glycoprotein and cartilage boundary lubricant, have been observed in the rat [20,21] and guinea pig [22] anterior cruciate ligament tear (ACLT) models, ovine meniscectomy model [23] as well as human ACLT [9]. Decreased lubricin concentrations in synovial fluid have been linked to inferior boundary lubricating properties [20,22,24].

In contrast, several studies evaluating synovial fluid composition following intra-articular fracture have found increased lubricin concentrations. Increased lubricin synovial fluid concentrations have been reported in horses with acute and chronic OA resulting from naturally occurring articular fractures [15,25], surgically-induced models of equine osteochondral fractures [25], human tibial plateau fractures [14] and advanced human OA [26]. In studies where lubrication was evaluated [14,15], boundary friction coefficients were elevated in synovial fluid from patients with intra-articular fracture. This phenomenon was attributed to decreased levels of HA and was supported by improved lubrication in vitro by supplementing synovial fluid with HA. Nevertheless, the mechanism by which lubrication changes may contribute to PTOA pathogenesis remains unclear. One limitation of most studies is that articular fracture location and the time interval between fracture and synovial fluid sampling was not uniform among subjects. Furthermore, only a single time point after injury was examined. A recent study measured serial synovial fluid lubricin concentrations in horses with surgically-induced osteochondral carpal fragmentation from onset of injury to seventy days post-injury [25]. Lubricin concentrations increased following intra-articular fracture, peaking at 14 days post-injury, and gradually returning to baseline by 70 days. In addition to permitting longitudinal synovial fluid sampling, the equine osteochondral fragment model is valuable for its clinical relevance to naturally occurring equine and human OA from injuries such as intra-articular fractures [14]. In addition, the equine model

has been extensively characterized and used to evaluate OA therapies [27]. While serial changes in lubricin levels have been reported in this model, it remains unknown how synovial fluid lubrication and other molecular constituents such as HA affect lubrication over time following intra-articular fracture.

Furthermore, several modes of cartilage lubrication occur throughout the gait cycle [24,28,29], but only boundary mode friction properties of synovial fluid have been characterized following articular fracture. Recently, multiple laboratories have applied the Stribeck framework to cartilage lubrication to map the friction coefficient of cartilage across these modes [24,30–33]. These studies have demonstrated that friction scales with sliding speed, viscosity, and inversely with contact load. The Stribeck framework was originally developed to describe the variation in friction coefficient in journal bearings from boundary mode where there is asperity-on-asperity contact to hydrodynamic lubrication in which surfaces are separated by a fluid film greater than the height of the asperities [29]. This method involves plotting the coefficient of friction as a function of a dimensionless Sommerfeld number (S) to create a "Stribeck curve", where S is calculated as: $S = \frac{v\eta a}{F_N}$ where v is sliding speed, η is viscosity, a is contact width (6mm), and F_N is normal load [24,29–31]. Unlike journal bearings, cartilage is soft and porous and likely unable to sustain a full fluid film. This results in friction coefficients greater than what would be expected for true fluid-film lubrication at high Sommerfeld numbers [24,29]. However, cartilage exhibits a similar decrease in friction coefficient with increasing Sommerfeld number, i.e., increasing speed or viscosity (Figure 2-3A) until a plateau in friction coefficient is met at high Sommerfeld numbers, which has been coined as "elastoviscous" lubrication mode [24,29]. The origins by which friction decreases is an active area of investigation with research supporting hypotheses such as

tribological rehydration [34,35], generation of a pressurized fluid wedge [30,34], and formation of viscous gel layers [24,29].

The Stribeck framework has proven to be a powerful tool to describe cartilage lubrication. Importantly, Stribeck analysis has shown that biochemical and mechanical damage to cartilage may result in failure of specific lubrication mechanisms as indicated by measurable changes in boundary and elastoviscous/minimum friction coefficients or transition numbers [30,31]. Likewise, changes in lubricant composition (lubricin and HA concentration, HA molecular weight) can dramatically affect the shape of the Stribeck curve and magnitude of friction within certain lubrication regimes. These changes are not detectable when friction is plotted versus only speed. Because increased friction can predispose cartilage to degradation in OA [6,20], it is important to understand how lubricant composition affects cartilage friction coefficients and the transitions from high-friction boundary mode to low-friction elastoviscous mode.

As such, there is a need to understand how articular fracture affects synovial fluid composition and its frictional properties across all modes of lubrication. This information could shed light on the role of lubrication in the pathogenesis of PTOA and reveal new approaches for treatment of articular fractures. Therefore, the goal of this study was to examine temporal changes in lubricin, HA, and synovial fluid lubrication over time in the equine carpal osteochondral fragment model of PTOA.

2.3 Methods

2.3.1 Osteochondral fragment model

Following approval by the Cornell University Institutional Animal Care and Use Committee, four Thoroughbred horses (n = 2 castrated males, n = 2 females) aged 2-6 years that were donated for research were used in this study. Horses were maintained in 3.65 m x 3.65 m box

stalls. In one randomly assigned limb of each horse, an 8mm osteochondral fragment was created under arthroscopic guidance in the middle carpal joint while the contralateral forelimb served as a sham-operated control [27]. Both limbs were irrigated using a polyionic balanced electrolyte solution (Plasmalyte, Baxter, Inc., Deerfield, IL). Synovial fluid samples were obtained from both limbs at the time of initial arthroscopy (day 0) and at days 5, 9, 12, 19, 26, 33, 61 and 75 post-operatively with aspiration volumes of approximately 2mL per joint as described previously [36]. Synovial fluid samples were centrifuged at 3,000 x g for 5 minutes immediately after collection, and synovial fluid supernatants were stored at -80°C until analysis. Prior to the surgery, each horse was acclimated to the treadmill for one to four days. Horses were exercised on a high-speed treadmill each morning five times weekly for the study duration beginning 2 weeks after surgery. Each session consisted of walking (5 km/h) for 5 min, followed by trotting (16-18 km/h) for 2 min, galloping (28-32 km/h) for 2 min, and ending with 2 min of trotting (16-19 km/h) exercise performed in the morning.

2.3.2 Biochemistry

Synovial fluid composition was analyzed at all time points. As described previously [25], synovial fluid was diluted 1:2,000 in PBS, and lubricin was quantified in duplicate using a peanut agglutinin sandwich ELISA with mAb 9G3 (Cat #: MABT401, Millipore Sigma, Burlington, MA) and purified equine synovial fluid lubricin as the standard. Lectin blots and immunoblots were performed on serial synovial fluid samples from one representative horse using biotinylated peanut agglutinin lectin (Vector Labs, Burlingame, CA), mAb 9G3, and a polyclonal anti-C-terminal lubricin antibody (Cat #: PA3-118, Thermo Fisher Scientific, Waltham, MA), which confirmed that the lubricin measured was full-length and was not degraded post-injury (Figure 2S- 2).

Hyaluronic acid concentrations were measured at all time points in duplicate using a commercially available HA ELISA (Hyaluronan DuoSet ELISA, Cat#: DY3614-05, R&D Systems, Minneapolis, MN) [37]. For the assay, equine synovial fluid samples from all horses at all time points were diluted 1:80,000 in 5% Tween 20 in PBS. Absorbance was measured at 450nm with wavelength correction set at 540nm.

HA molecular weight distribution was determined for all time points by gel electrophoresis similarly to a previously described method [38]. Equine SF samples were diluted 1:20 in PBS and were treated overnight with 70μg/mL proteinase K (Proteinase K, recombinant, PCR grade, Roche Applied Science, Mannheim, Germany). Synovial fluid samples were loaded onto 1% agarose gels. HA standards, Select-HA HiLadder (0.5-1.5 MDa) and Mega-HA Ladder (1.5-6.1 MDa; AMS Biotechnology Limited, Cambridge, MA), were loaded onto each gel to characterize the molecular weight distributions of HA. Electrophoresis was conducted at 50V for 8 hours, and gels were stained overnight with 0.005% Stains-All (Sigma-Aldrich, St. Louis, MO) in 50% ethanol. The following day, gels were de-stained in a 10% ethanol solution for 24 hours. Gel images were acquired using a Bio-Rad VersaDoc Imaging System (Hercules, CA), combined using the Stitching plugin [39], and relative band intensity was quantified in ImageJ.

2.3.3 Lubrication Analysis

To determine the frictional properties of synovial fluids, samples were tested on a custom tribometer as previously described [32]. Briefly, cylindrical cartilage explants (6mm diameter x 2mm thickness) were harvested from the patellofemoral groove of neonatal bovine stifles and loaded onto a tribometer in a bath of synovial fluid. Synovial fluid from both sham and injured limbs at days 0, 19, 33, and 61 was tested due to an insufficient volume remaining for analysis at other time points. Explants were compressed to approximately 40% strain against a glass counter-

face and permitted to depressurize over the course of one hour. After reaching an equilibrium normal load, the counter-face was linearly reciprocated at speeds ranging from 0.1-10mm/s for 2-3 cycles per speed. Simultaneously, a biaxial load recorded the normal and shear loads. For both the forward and reverse directions and at each speed, the friction coefficient was calculated as the mean shear force at the end of sliding when friction had reached an equilibrium value divided by the equilibrium normal load. Care was taken to minimize fluid evaporation during the test, and once the test was completed, synovial fluid was collected and stored at -80°C before evaluating viscosity.

After friction testing, viscosity measurements were performed on the same synovial fluid samples using a commercial rheometer (TA Instruments DHR3 Rheometer, New Castle DE). A flow sweep test where angular velocity varied from 0.1-100 rad/s was performed using a 20mm parallel plate fixture and gap distance of 500μm to determine viscosities of synovial fluids from sham and injured limbs. Only synovial fluid collected on days 0, 19, 33, and 61 were tested due to an insufficient volume for analysis at other time points. Viscosity measurements presented are for shear rates of 2 Hz or 0.1rad/s, which is generally within synovial fluid's first Newtonian plateau and has previously been shown to appropriately scale Stribeck curves [24].

2.3.4 Stribeck Analysis

Lubrication and rheological data were fit to a dimensionless Sommerfeld number, S,

$$S = \frac{v\eta a}{F_N}$$

where v is sliding speed, η is viscosity, a is contact width (6mm), and F_N is normal load [24,29]. Stribeck curves were generated by plotting friction coefficient as a function of Sommerfeld number. Transition number, S_t, was calculated by fitting friction coefficients from speeds 0.1-

1mm/s, i.e., during the mixed lubrication mode, vs. respective Sommerfeld number to the equation [24]:

$$\mu = \mu_{min} + (\mu_B - \mu_{min})e^{-S/S_t{}^d}$$

in MATLAB using a linear least-squares method (MathWorks, Natick, MA). The boundary friction coefficient, μ_B, was taken from the Stribeck curves as the upper plateau in friction coefficient at v=0.1mm/s and the minimum or elastoviscous mode friction coefficient, μ_{min}, was taken as the lower plateau in friction coefficient at v=5-10mm/s.

2.3.5 Statistics

All data was analyzed using a linear mixed effects model to account for the hierarchical nature of the data. The fixed effects in the model included treatment (sham vs. OA limb), day, and a treatment·day interaction term. Random effects included horse and horse·treatment to account for the non-independence of observations. To satisfy model assumptions and normalize data, square root values were used for lubricin ELISA, transition number, and HA 0.5-1.5MDa gel data, and logarithmic values were used for HA ELISA data. All summary statistics and values shown in figures represent un-transformed data with mean ± SEM. In the case of significant fixed effect terms, post-hoc analysis was performed using Dunnett's test to compare to day 0 baseline values. P-values < 0.05 were considered significant. Statistical analysis was performed using JMP Pro 13.0 software (SAS Institute Inc., Cary, NC).

2.4 Results

Osteochondral fragmentation resulted in significant changes in the composition of synovial fluid with respect to lubricin, HA, and HA molecular weight distribution. HA concentrations decreased significantly after fracture in both the sham and OA limbs (Figure 2-1B), with day 5 concentrations approximately 80% lower than day 0 baseline values. Concentrations remained

significantly decreased relative to day 0 baseline values until day 33 after surgery. Similarly, high molecular weight HA (>6.1MDa, Figure 2-1C) was significantly lower relative to day zero baseline for all time points after injury in both the sham and OA limbs. The 3.1-6.1MDa and 1.5-3.1MDa HA concentrations (Figure 2-1D,E) were likewise decreased following injury, but recovered to values similar to baseline by day 61 and day 75, respectively. No changes in the concentration of low molecular weight HA (0.5-1.5MDa, Figure 2-1F) were observed. Lubricin concentrations increased in both OA and sham limbs post-operatively, peaking at 2-3 weeks after injury and remaining elevated until day 61 (Figure 2-1A). Lubricin concentrations were approximately 2-3x higher in injured than control limbs ($p=0.008$).

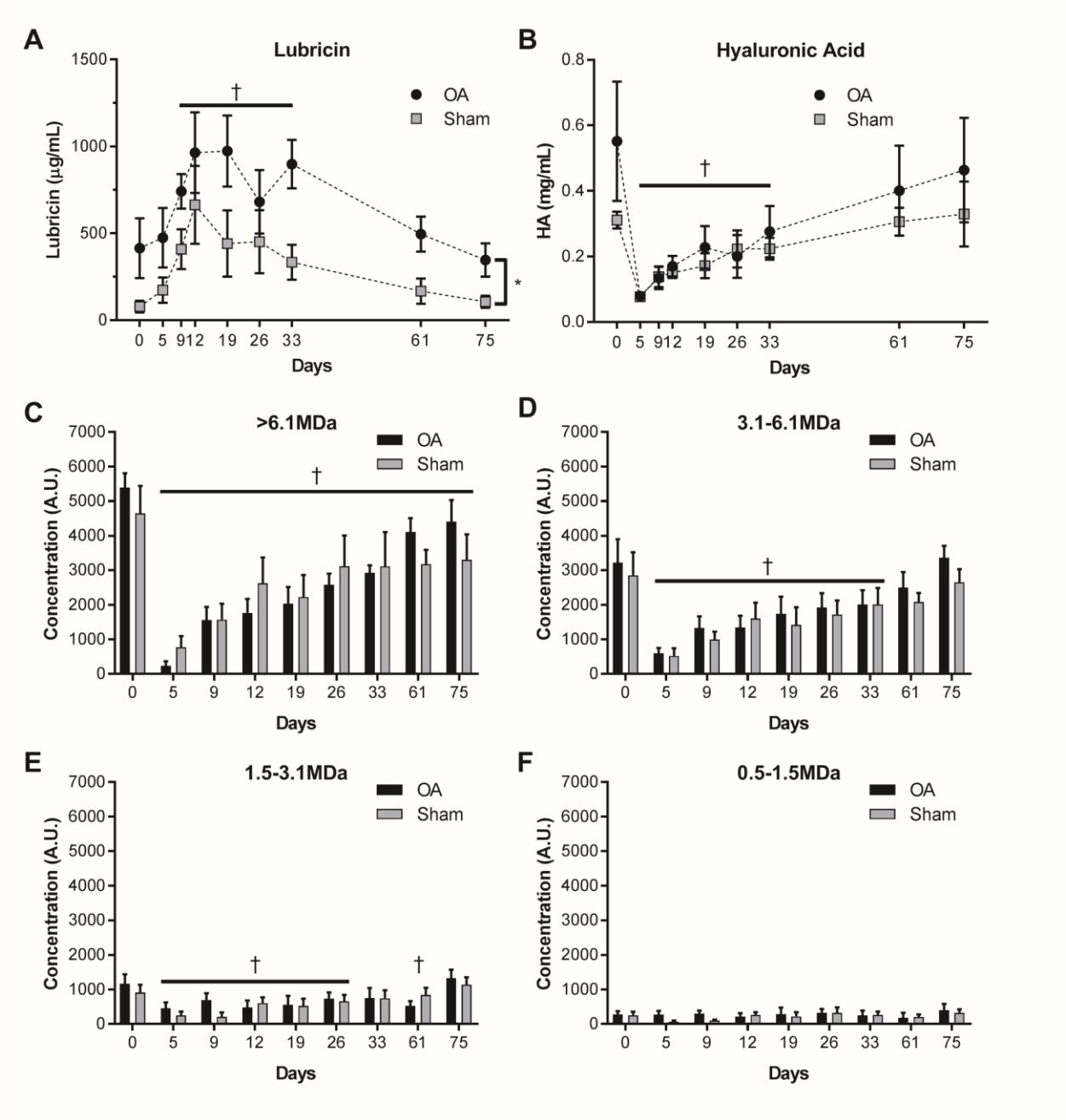

Figure 2-1: Synovial concentration of (a) lubricin increased after injury with levels peaking at 2-3 weeks after injury and not returning toward baseline until day 61 after injury. In contrast, the total concentration of (b) HA decreased after injury, especially the (c) high molecular weight fraction (>6.1MDa). For the >6.1MDa fraction, synovial concentrations of HA did not recover to baseline values over the course of the study while the 3.1-6.1MDa fraction did not

recover until 61 days after surgery. Note that † indicates that the combined sham and OA data are significantly different than day 0 at the specified timepoints and * indicates a significant difference between treatment groups across timepoints ($p<0.05$, $n=4$).

Lubrication analysis revealed distinct changes in synovial fluid with respect to viscosity and lubrication modes, but no significant differences were observed between sham and OA limbs for these measurements ($p>0.05$). Compared to baseline values, low shear rate viscosity decreased by nearly half by day 19 and remained significantly lower at days 33 and 61 (Figure 2-2, $p<0.01$). Surprisingly, boundary friction and minimum friction coefficients did not vary over time nor differ between control and OA limbs (Figure 2-3B,D). Rather, a distinct shift in transition number was observed; in both injured and sham limbs, the transition number was significantly lower at all time points after surgery indicating an earlier transition from boundary mode toward elastoviscous mode as compared to baseline values (Figure 2-3C).

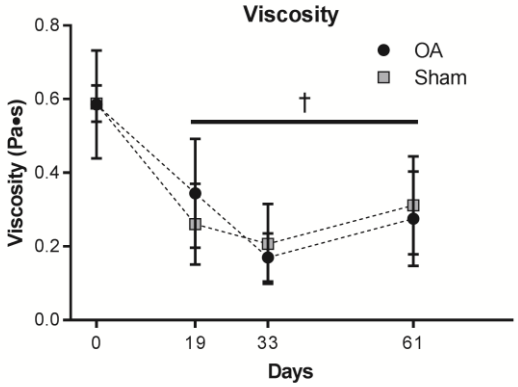

Figure 2-2: Viscosity decreased after injury and did not recover to baseline values. Note that † indicates that the combined sham and OA data are significantly different than day 0 at the specified timepoints ($p<0.05$, $n=4$).

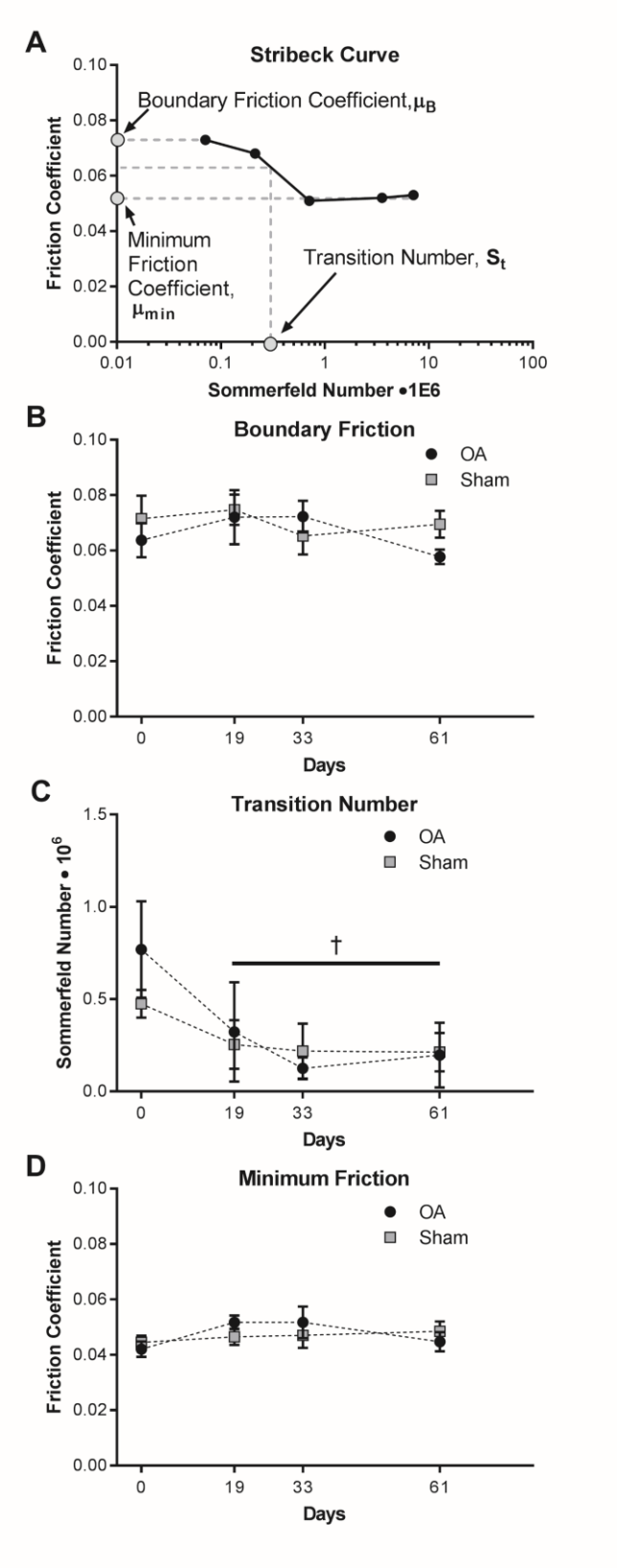

Figure 2-3: (A) Stribeck curves were created by plotting friction coefficients as a function

of the Sommerfeld number, which factors in the viscosity of synovial fluid and tribological test parameters including sliding speed, contact width, and normal load. Stribeck curves were used to analyze how lubrication changed over time with respect to (B) boundary lubrication and (D) elastoviscous lubrication and the (C) transition point between these modes. Note that † indicates that the combined sham and OA data are significantly different than day 0 at the specified timepoints ($p<0.05$, $n=4$).

2.5 Discussion

Synovial fluid lubricin, HA, and HA molecular weight distribution were significantly altered following OA induction via osteochondral carpal fracture in the equine model. Interestingly, a reduction in transition number and synovial fluid viscosity were observed after surgery while boundary and minimum friction coefficients remained unchanged. Similar to past results in this model [25] and in human and equine articular fractures [14,15], lubricin concentrations were significantly higher after injury. Interestingly, although lubricin concentrations were higher in the limb undergoing fracture, derangements in lubricin, HA and HA molecular weight distribution were paralleled between the injured and sham-operated limbs. This suggests that the loss of HA may be more significantly affected by synovial fluid washout during the arthroscopic procedure as opposed to the carpal fragmentation injury, whereas lubricin expression is induced by intra-articular fragmentation. Total HA concentrations as quantified via ELISA were significantly reduced relative to baseline for four weeks after injury. Notably, the high molecular weight HA fractions as quantified via gel electrophoresis were particularly disturbed after fracture; for the highest molecular weight forms (>6.1MDa), levels remained below baseline for all time points measured after injury (10 weeks) while the 3.1-6.1MDa fraction

remained low for four weeks after injury. A concurrent decrease in viscosity of synovial fluid was observed.

With respect to lubrication, no changes in boundary mode or elastoviscous mode friction coefficients were observed over time nor between sham-operated and injured limbs. We have previously determined that maximal boundary lubrication on our custom tribometer was achieved at a concentration of approximately 40μg/mL of recombinant human lubricin, with concentrations up to 350μg/mL having no additional effects on friction coefficient [40]. Here, we used similar testing conditions, and thus attribute the lack of change in boundary friction coefficients to synovial lubricin concentrations far exceeding 40μg/mL in the present study. Dose-response relationships between lubrication and lubricin concentration vary among reported studies and may be affected by lubricin source, lubricin quantification methods, and tribometer setup (cartilage-on-cartilage vs. cartilage-on-glass). Notably even between different instruments, saturating concentrations range from 40-150μg/mL lubricin, but friction coefficients at saturation converge toward ~0.10 [40,41]. This convergence suggests that the median effective dose of lubricin is especially dependent on lubricin source and quantification. Importantly, the dose-response relationship for lubrication of equine cartilage by lubricin has not been previously reported.

Furthermore, the lack of change in elastoviscous friction coefficients despite the decrease in viscosity was surprising. In hard, non-porous materials, low friction coefficients occur as a fluid film develops between articulating surfaces, and this fluid film is promoted by lubricant viscosity. However, for soft, porous materials like cartilage, full fluid-film lubrication is likely not achieved due to fluid flow back into the cartilage matrix and deformation of cartilage asperities [24,29,42]. Still, reduced friction with increased sliding speed may be explained by partial fluid film lubrication [29], tribological rehydration [35], and generation of pressurized fluid wedges [30,42].

Therefore, we posit two theories as to why elastoviscous friction coefficients did not vary; first, viscosity may play competing roles in lubricating the tissue. While higher viscosity fluids may better support partial fluid film lubrication, tribological rehydration may be hindered due to a reduced ability to flow. Alternatively, minimum friction coefficients may be limited by the properties of cartilage tissue, including its roughness and permeability. Our results agree with a previous study in which viscosity was varied over several orders of magnitude [24]. In previous studies of healthy cartilage, similar values of μ_{min} values were observed when lubricant composition was varied [24]. In contrast, an increase in μ_{min} was associated with cartilage impact and surface roughening suggesting that cartilage properties may be crucial to lubrication in this regime [30]. Overall, the lack of change in elastoviscous friction coefficients observed despite the decrease in viscosity warrants further investigation into the mechanisms of elastoviscous lubrication.

We observed the most dramatic changes in lubrication in the mixed lubrication regime. Interestingly, the transition number decreased after surgery in both limbs, indicating an earlier transition away from high-friction boundary mode. The effects of lubricin-HA interactions on Stribeck behavior has only recently begun to be investigated [24], and more information is necessary to understand how varying concentrations and overall lubricant viscosities affect cartilage friction regimes. Numerous studies have suggested that lubricin and HA function synergistically to lubricate cartilage [24,43]. Although a dose-response Stribeck analysis of lubricin and HA has not yet been conducted, a recent study from our lab has found that both boundary friction coefficients and the transition number were reduced when lubricin was added with HA as compared to HA alone [24]. This observation is consistent with the idea that lubricin-

HA interactions are facilitated by the elevated lubricin concentrations observed after injury. Such interactions may enhance lubrication through a shift to lower friction at lower sliding speeds.

In contrast to our results, similar studies of synovial fluid after articular fracture have observed greater boundary friction coefficients. In other studies where increased synovial fluid lubricin concentrations were also observed [14,15], greater boundary friction coefficients were reported, leading authors to hypothesize that decreased HA concentrations and a loss of high molecular weight HA may increase friction. In the acute post-injury phase (<3 weeks), Antonacci et al. and Ballard et al. reported a 39% and 100% increase, respectively, in boundary friction coefficients. We similarly observed decreased HA and increased lubricin levels after injury, but no significant changes in boundary lubrication were observed (<8%). The overall magnitude of boundary friction coefficients observed in our study averaged ~0.07, while Ballard et. al. and Antonacci et al. reported a range of 0.022 for control synovial fluid to a maximum of 0.044 for injured synovial fluid. One challenge in comparing our results to previous studies is that differing types of tribometers were used; while only stationary contact area configurations were used, here, we employed a cartilage-on-glass system with linear reciprocating motion whereas others used a cartilage-on-cartilage framework with a rotational motion [14,15]. Recent investigations using a rotational tribometer have found that boundary lubrication by HA is dependent on the counterface material; relative to saline, a reduction in friction coefficient by HA was only observed in cartilage-cartilage interfaces as compared to cartilage-glass interfaces [44]. Notably other studies have demonstrated that HA and other viscous lubricants do indeed lower friction coefficient in cartilage-on-glass systems [24,30,31,33]. Furthermore, the Stribeck framework was not applied in those studies. The Stribeck framework is advantageous because it elucidates the friction regime experienced by cartilage while controlling for viscosity, normal load, and sliding speed, which can

shift the system between boundary and elastoviscous friction modes. In other studies [14,19], marked differences in HA molecular weight distribution and concentration, a major driver of synovial fluid viscosity, were reported, but the synovial fluid viscosity was not investigated and only a single, slow rotational speed (0.3mm/s) was used. Thus, it is possible that the boundary friction coefficients we have reported (at linear speeds of 0.1mm/s) may not be directly comparable due to differences in testing equipment and definition of boundary regime.

Importantly, HA was significantly diminished after fracture and remained depleted long after injury. This occurred not only in the fractured joint but also, albeit to a lesser extent, in the contralateral sham-operated joint. It is intuitive that HA concentrations would be decreased following arthroscopy and saline lavage. Recent studies have demonstrated that lavage reduces lubricin staining at the cartilage surface [45] [46] and results in impaired boundary friction properties of cartilage [45,46]. However, it was surprising that the loss of high molecular weight HA was sustained for so long following fracture, especially given how quickly lubricin concentrations recovered. One possible explanation is that horses returned to intense treadmill exercise 2 weeks following injury, which may have been a persistent stimulus for HA degradation, impaired HA synthesis or loss of HA due to increased synovial membrane permeability [47]. Additionally, the contralateral limb could be affected by systemic effects of the injury or compensatory overloading to protect the injured limb. Further studies are necessary to explore how HA viscosupplementation after articular fracture or arthroscopic lavage may affect synovial fluid lubrication and osteoarthritis development. In an in vitro test, boundary lubrication was restored to normal values when high molecular weight HA was added to synovial fluid samples that showed impaired boundary lubrication and low HA levels [15]. Other clinical studies have reported improvements

in patient pain scores and joint swelling [48] in patients that received viscosupplementation after partial meniscectomy.

Additionally, the elevated lubricin concentrations we observed after injury contribute to a growing body of evidence that lubricin may be modulated differently by species, injury type, exercise, or other factors. Decreased lubricin levels have been reported after anterior cruciate ligament (ACL) tears in humans [9], rat models of ACL tear [8], and guinea pig ACL tear models [22] relative to controls. In contrast, in other end-stage human OA studies [26], in an ovine ACLT model [19] and equine [15,25] and human [14] articular fractures, synovial fluid exhibits elevated lubricin concentrations. Several assays, including ELISAs [8,9,22,25,26] and semi-quantitative western blots [14,15,22,26] have been used to measure lubricin, which could also explain the variations between studies. Most small animal destabilization models of OA have reported decreased synovial fluid lubricin, while increases have been mostly reported in large animal and human cases of articular fracture. Lubricin expression is known to be modulated by exercise [21], mechanical loading [49], and cytokines like TGF-β, TNF-α and IL-1β [7,50–52] . Furthermore, mesenchymal stem cells (MSCs) are known to produce copious amounts of lubricin [53,54] and, as such, their release into the joint space due to the osteochondral fragment induction may have led to increased lubricin release into synovial fluid. As many research groups are investigating the therapeutic potential of lubricin to treat osteoarthritis, it is important to understand when it may be indicated for treatment.

This study was not without limitations. Only four horses were evaluated during this study. However, the linear mixed model accounted for serial measurements from each horse, which increased the power of our statistical comparisons. Furthermore, we did not examine expression and enzymatic activity within the joint that resulted in alterations in lubricin and HA

concentrations or the specific biological activity of these molecules. Both molecules have demonstrated anti-inflammatory properties [55] and may play important roles in joint homeostasis distinct from their roles in lubrication. Finally, this study used healthy neonatal bovine tissue in combination with the equine synovial fluid samples for measuring lubrication. While this was chosen to enable serial measurements of synovial fluid lubrication throughout OA development, we did not examine the frictional changes in the equine cartilage tissue. Hallmarks of the equine osteochondral fragment model and OA including proteoglycan loss and surface fibrillation have been associated with inferior tribological properties [30,31,56].

In conclusion, we have shown distinct temporal changes in the concentrations of the critical synovial fluid lubricants, HA and lubricin, in an equine experimental OA model. Importantly, concentrations of HA, especially high molecular weight forms, decreased significantly after surgical intervention and remained below pre-surgery baseline values for weeks after injury. Lubricin concentrations increased after articular fracture in agreement with previous studies in this model and other cases of articular fracture. Interestingly, no changes were observed in boundary mode friction or elastoviscous mode friction coefficients. However, the transition number representing the shift between high-friction boundary mode and low-friction elastoviscous mode was reduced after surgery. This suggests that lubrication was enhanced after fracture. Notably, the sham-operated limb exhibited a similar magnitude of concentration changes in lubricin and HA and lubricating properties as compared to the control limb. This suggests that non-operated animals or non-operated contralateral limbs should be included in studies using this experimental model that aim to evaluate lubrication outcome parameters. Future studies should focus on elucidating the biological and mechanical roles of lubricin and HA. Despite the wide variation of lubricin and HA in disease, frictional changes, and especially multi-modal friction changes, have been

examined for only a narrow subset of concentrations. Altogether, this study indicates that there are distinct time-dependent changes in synovial fluid lubricant composition and frictional properties following articular fracture.

2.6 Acknowledgements

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2.7 Supplemental Figures

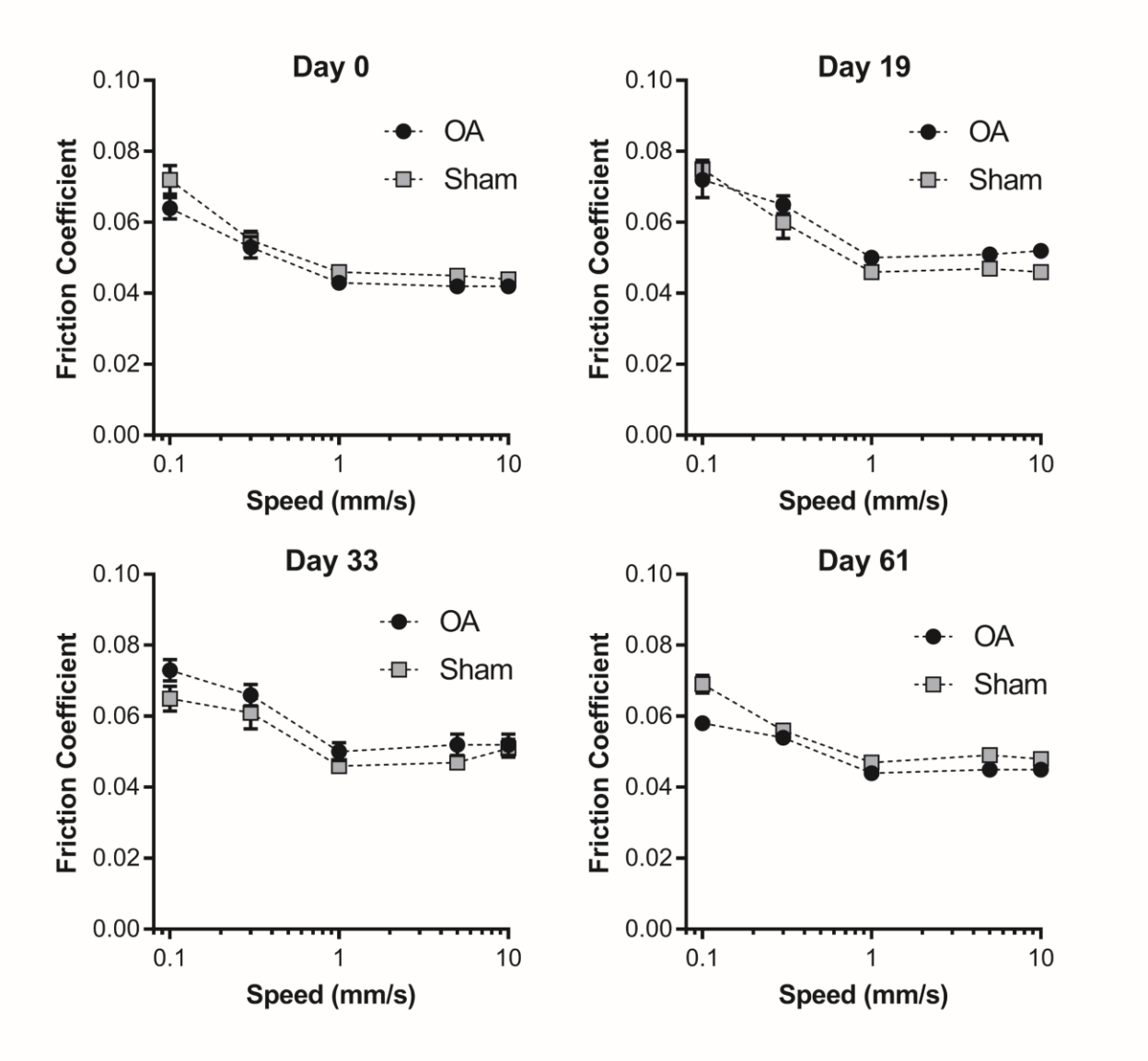

Figure 2S- 1: Friction versus sliding speeds show similar behavior between OA and sham limbs (n-4).

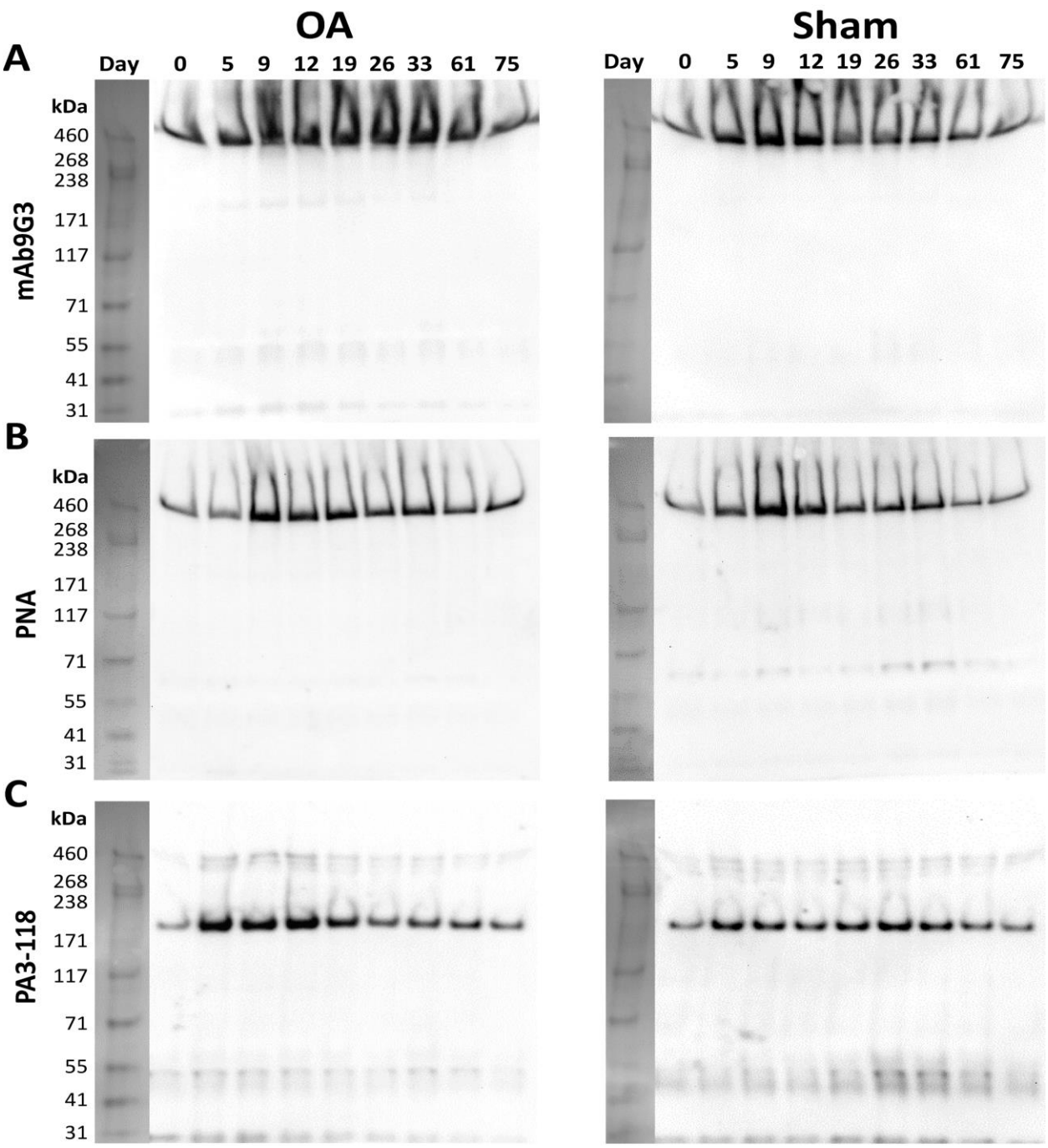

Figure 2S- 2: The presence of full-length lubricin was confirmed by lectin blots and immunoblots performed on serial synovial fluid samples from one representative horse using (A) mAb9G3 (MABT401, 1:1000 dilution), (B) biotinylated peanut agglutinin lectin (1:1000 dilution), and (C) a poly-clonal anti-C-terminal antibody (PA3-118, 1:700 dilution).

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CHAPTER 3 [1]

Inflammatory and Non-Inflammatory Synovial Fluids Exhibit New & Distinct Tribological Endotypes

3.1 Abstract

Inferior synovial lubrication is a hallmark of osteoarthritis (OA), and synovial fluid (SF) lubrication and composition are variable among OA patients. Hyaluronic acid (HA) viscosupplementation is a widely used therapy for improving SF viscoelasticity and lubrication, but it is unclear how the effectiveness of HA viscosupplements varies with arthritic endotype. The objective of this study was to investigate the effects of the HA viscosupplement, Hymovis®, on the lubricating properties of diseased SF from patients with non-inflammatory OA and inflammatory arthritis (IA). The composition (cytokine, HA, and lubricin concentrations) of the SF was measured as well as the mechanical properties (rheology, tribology) of the SF alone and in a 1:1 mixture with the HA viscosupplement. Using rotational rheometry, no difference in SF viscosity was detected between disease types, and the addition of HA significantly increased all fluids' viscosities. In non-inflammatory OA SF, friction coefficients followed a typical Stribeck pattern and their magnitude was decreased by the addition of HA. While some of the IA SF also showed typical Stribeck behavior, a subset showed more erratic behavior with highly variable and larger friction coefficients. Interestingly, this aberrant behavior was not eliminated by the addition of HA, and it was associated with low concentrations of lubricin. Aberrant SF exhibited

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significantly lower effective viscosities compared to non-inflammatory OA and IA SF with typical tribological behavior. Collectively, these results suggest that different endotypes of arthritis exist with respect to lubrication, which may impact the effectiveness of HA viscosupplements in reducing friction.

3.2 Introduction

Osteoarthritis (OA) is characterized by a loss of synovial fluid lubrication that results in higher strains on the tissue and promotes articular cartilage degradation [1,2], chondrocyte death [3], mitochondrial dysfunction [4], and apoptosis [5,6]. In a healthy joint, cartilage tissue exhibits low friction in part due to the synovial fluid bathing the tissue. In OA, synovial fluid exhibits increased friction coefficients and a reduced viscosity that are associated with fluctuations in the concentrations of essential lubricating molecules, the glycoprotein lubricin [7,8] and hyaluronic acid (HA). As such, treatments to improve lubrication are of great interest. Intra-articular injection of exogenous HA into the joint space, i.e., viscosupplementation, has been used clinically for decades to treat OA. Viscosupplements act both mechanically and biologically within the joint space [9–11]. Delivery of high molecular weight HA increases the viscosity of the synovial fluid, which results in improved lubrication. In vitro studies have demonstrated that HA also exerts anti-inflammatory and analgesic effects as well as stimulates endogenous HA production [11,12]. Despite these benefits, prominent orthopedic associations offer conflicting guidelines to clinicians about the use of HA [13–16]. The precise mechanism of action of viscosupplements is poorly understood, and clinical results vary widely. Several factors have been associated with affecting the efficacy of HA viscosupplementation such as patient age [17], obesity [18,19], joint effusion [20], severity of OA [17,21,22], and the location of cartilage degeneration [20]. Furthermore,

viscosupplements' clinical benefits have been correlated with their ability to lubricate cartilage [23].

A challenge in tailoring OA treatments is that OA is a family of diseases influenced by genetics, aging, chronic and acute mechanics, biochemistry, and other factors [24–26]. While long-term outcomes are similar, the mechanisms differ and may be amenable to unique treatments. Indeed, there is a growing appreciation that there exist many phenotypes and endotypes of osteoarthritis [27]. Recent research has focused on identifying OA phenotypes by profiling patients' blood serum [28], urine [29,30], and synovial fluid [31–33]. These analyses have distinguished healthy patients and OA and rheumatoid arthritis patients [32,33], progressive and non-progressive OA in obese patients [30], and stages of OA [32] based on differences in metabolites (i.e., chondroitin sulfate degradation [31], lipid types [32], and cytokine levels [30]). Altogether, a predictive factor to identify responders to viscosupplementation remains elusive.

Importantly, few studies have examined how synovial fluid mechanics vary with OA type. Most studies of synovial fluid lubrication and composition characterize post-traumatic OA showing that changes in the fluid are highly dependent on the injury and amount of time after the initial injury [34–37]. For example, relative to normal levels, lubricin concentrations increased following articular fracture while concentrations decreased following anterior cruciate ligament tears [35,36,38]. HA concentrations, especially the high viscosity or high molecular weight (MW) polymer, have been found to decrease in OA in humans and large animals and vary temporally [35,36,39–44]. Synovial fluid collected from unique populations of arthritis patients exhibit different ex vivo tissue shear strains [45]. Collectively, these observations have important consequences on the treatment of OA as they suggest that lubrication-based therapies may benefit certain populations of patients based on their synovial fluid properties.

One major distinction used to classify arthritis is by whether it shows non-inflammatory or inflammatory features, which is determined by the amount of white blood cells in the synovial fluid [46,47]. The lubricating properties of synovial fluid from these cohorts is unknown, and it is unclear if and how viscosupplementation with HA may affect lubrication. Therefore, the objective of this study was to investigate the differences in the lubricating properties of synovial fluid from two different populations of patients with either non-inflammatory OA or inflammatory arthritis. Secondary to this aim, we investigated how supplementation with the modified HA viscosupplement, Hymovis®, affected lubrication in these cohorts.

3.3 Methods

3.3.1 Sample Collection + Preparation

The Institutional Review Board at the University of Padua granted approval for collection and storage of human synovial fluids (Protocol Number: 0039872/2015). Synovial fluid (SF) was collected with patient consent from the knee joints of donors receiving therapeutic arthrocentesis for joint effusion at their initial presentation to the clinic or in response to an arthritic flare. A total of twenty patients without a history of joint trauma provided synovial fluid samples (aged 21 to 90 years old, 15/20 female, 5/20 male). The volume aspirated ranged from 4-160ml with a mean value of 40.5ml and median value of 27.5ml. After aspiration, a subset of the fluid was analyzed by optical light microscopy; a hematological counting chamber was used to determine the total white blood cell (WBC) count, and the polymorphonuclear neutrophil (PMN) composition was determined by supravital staining [48]. The remaining SF was centrifuged at 3,000 RPM for ten minutes to remove cellular contents and debris and then stored at -80°C. The WBC data were used to classify arthritis type; inflammatory synovial fluids (n=10, 7/10 female, 5/10 receiving therapeutic arthrocentesis at their initial visit and 5/10 in response to a flare) were defined as having

WBC counts exceeding 2,000/mm^3 while non-inflammatory synovial fluids (n=10, 8/10 female, 4/10 receiving therapeutic arthrocentesis at their initial visit and 6/10 in response to a flare) had WBC counts below this threshold [49]. The inflammatory arthritis patients within this study were further diagnosed by a licensed physician with one of the following arthritis sub-types: crystal-induced arthritis (n=2), early arthritis (n=2), psoriatic arthritis (n=2), reactive arthritis (n=3), and spondyloarthritis (n=1) [50–52]. WBC count data for individual patients and group means can be found in Table 3S- 1.

3.3.2 Lubrication Analysis

The mechanical properties of the synovial fluids were evaluated either alone or in a 1:1 mixture with Hymovis®. Hymovis® is based on a modified HA in which a linear hexadecyl (C_{16}) side chain is grafted along the HA backbone at a degree of substitution of approximately 3% [53]. These sidechains enable hydrophobic aggregation and thus produce a high effective viscosity of approximately 70 Pa·s [54]. Mixtures were prepared by combining equal volumes of HA and synovial fluid and vortexing for approximately 30s. The lubricating properties were evaluated using a cartilage-on-glass tribometer, rheometry, and the Stribeck framework. Results from both cartilage-on-glass tribometry and the Stribeck framework have previously been used to characterize synovial fluids [44] and to relate lubricating properties of viscosupplements to clinical outcomes [23].

3.3.2.A Tribology

To determine the lubricating properties of the synovial fluids and viscosupplement mixtures, a custom cartilage-on-glass tribometer was used as previously described [55]. Briefly, cylindrical cartilage explants (6mm diameter x 2mm thickness) were harvested from the femoral condyles of neonatal bovine stifles and loaded into a synovial fluid bath on the tribometer. Explants

were compressed against a glass counter-face under 40% axial strain. After reaching an equilibrium normal load approximately one hour after initial compression, the counter-face was linearly reciprocated at speeds ranging from 0.1-10mm/s for 2-3 cycles per speed. Simultaneously, a biaxial load recorded the normal and shear loads. For both the forward and reverse directions and at each speed, the friction coefficient was calculated as the mean shear force at the end of sliding when friction had reached an equilibrium value divided by the equilibrium normal load. Care was taken to minimize fluid evaporation during the test by covering the synovial fluid baths.

3.3.2.B Rheology

Viscosity measurements were performed using a commercial rheometer (Anton Paar MCR 702). A flow sweep test was performed using a 25mm truncated cone-and-plate fixture with a 2° cone angle. Angular velocity was varied from 1000-1x10^{-4} s^{-1} to determine viscosities of synovial fluids and synovial fluid-HA mixtures [23,44,54].

3.3.2.C Stribeck Analysis:

Sommerfeld numbers, *S*, were calculated as:

$$S = \frac{\eta v a}{F_N} \quad (1)$$

where v is sliding speed, η is viscosity, a is contact width (6mm), and F_N is normal load. Stribeck curves were generated by plotting friction coefficient as a function of Sommerfeld number. Viscosity values used to calculate *S* were measured via rotational rheometry at a shear rate of 0.1 s^{-1} [44]. Supplemental Figure 3S- 1 shows the friction coefficient versus sliding speed curves for each synovial fluid sample.

As described in previous studies [23,44,54], synovial fluid friction data were fit to an idealized Stribeck curve:

$$\mu(S) = \mu_{min} + (\mu_B - \mu_{min}) e^{-(S/S_t)^d} \quad (2)$$

Where μ_{min} is the minimum friction coefficient, μ_B is the boundary friction coefficient, S_t is the Sommerfeld number at the inflection point between boundary and elastoviscous modes, and d is a fitting parameter controlling the slope of the transition between modes.

In addition to using measured viscosities to calculate S, an *effective viscosity* was calculated for each lubricant, which has been shown to correlate with viscosupplements' clinical outcomes [23]. Furthermore, effective viscosity has been used for generating Stribeck curves of shear-thinning materials in soft contacts [56–59], similar to synovial fluid and our tribological test setup in which the exact shear rate of the fluid between the cartilage-glass interface is unknown. Here, effective viscosity calculation was performed by first calculating a predicted Sommerfeld number via Eq. (1) in which viscosity, η, could vary. A theoretical friction coefficient, μ, was calculated using Eq. (2) with the coefficients set as μ_{min}=0.04, μ_B=0.15, S_t=2.7E-6, d=0.62, and S as the predicted Sommerfeld number to represent a model Stribeck curve [23,54]. Then, the root mean square (RMS) error between the measured and theoretical friction coefficients was minimized by allowing viscosity to vary, thus yielding an effective lubricating viscosity. Information on the RMS errors are available in Supplemental Table 3S- 3 and Table 3S- 4.

3.3.3 Synovial Fluid Biochemical Analysis

For all biochemical assays performed, the volume of synovial aspirated for each patient was not factored into the analysis.

3.3.3.A Lubricin Quantification

As previously described [34], synovial fluid was diluted 1:3,200 and 1:6,400 in PBS, and lubricin was quantified in triplicate using a peanut agglutinin sandwich enzyme-linked immunosorbent assay (ELISA) with mAb 9G3 (Millipore Sigma, Burlington, MA) and purified bovine synovial fluid lubricin as the standard. Similar concentrations of lubricin were found

between the two dilutions, and the 1:3,200 dilution measurements were arbitrarily chosen for presentation in the figures. The mean intra-assay coefficients of variation were 2.5±1.1% and 3.8±2.5%.

3.3.3.B HA Quantification

Synovial fluids were diluted to 1:80,000 and 1:100,000 and HA concentrations were quantified in triplicate using a commercial sandwich ELISA (R&D Systems, Minneapolis, MN) per manufacturer instructions. The 1:80,000 dilution measurements were chosen for presentation in the figures due to a lower coefficient of variation among replicates as compared to the 1:100,000 dilution. The mean intra-assay coefficients of variation were 5.9±4.1% and 4.6±2.1%.

3.3.3.C Cytokine Profiling

Synovial fluid centrifugation was performed again prior to cytokine analysis via ELISA. Samples were diluted as necessary, and the following cytokines were measured in duplicate: IL-1ß, CCL2, IL-6, IL-8, IL-10, and TGF-ß using commercial kits (ThermoFisher Scientific, Waltham, MA). Intra-assay coefficients of variation were below 9%.

3.3.4 Statistics

For all statistical tests, a p-value less than 0.05 was considered a statistically significant difference. The results of all statistical tests are available in Supplemental Table 3S- 5. Measured viscosities at shear rates of 0.001-100 s^{-1} and effective viscosities were compared among groups by applying a mixed linear model in RStudio using the *lmerTest* package and *emmeans* packages [60,61]. Fixed effects of disease type (OA, inflammatory arthritis (IA), and for effective viscosities, IA with aberrant tribology), presence of HA, and their interaction were specified in addition to a random effect of subject. A log or inverse transformation was performed as necessary to satisfy models' assumptions of normality. Post-hoc tests with Tukey or Sidak corrections were

performed to detect significant differences and correct for multiple comparisons. The Kenward-Roger method was used to calculate the degrees of freedom.

A Kruskal-Wallis test by ranks was performed followed by Dunn's post-hoc tests with Bonferroni corrections to compare cytokine concentrations, and HA and lubricin concentrations among groups.

The difference in friction coefficients from 0.1 to 10mm/s, Δμ, were compared using an unpaired t-test with Welch's correction for inequal variances, and friction coefficients collected at 0.1mm/s sliding speed were fit to a one-phase decay model with lubricin and HA concentrations (defined as *x*) as follows:

$$\mu(x) = (\mu_o - \mu_{min})e^{-kx} + \mu_{min} \quad (2)$$

Previous dose-response studies of lubricin on friction coefficient have fit the data to a four-parameter logistic model [62]; in the present study, a high-friction plateau was not observed and so the one-phase decay model was used as an approximation to relate friction to lubricin and HA.

3.4 Results

With respect to rheological properties of the synovial fluids, inflammatory and non-inflammatory synovial fluids exhibited similar mean viscosities across all shear rates (Figure 3-1A). The addition of HA significantly increased the fluids' viscosities, especially at low shear rates (Figure 3-1B-F, p-values ranged from 0.0001 to 0.05, n_{IA}=7-10, n_{IA+HA}=9, n_{OA}=7-9, n_{OA+HA}=8-9); the fold increase in mean viscosity ranged from 18- to 90-fold for shear rates below 0.01 s^{-1}.

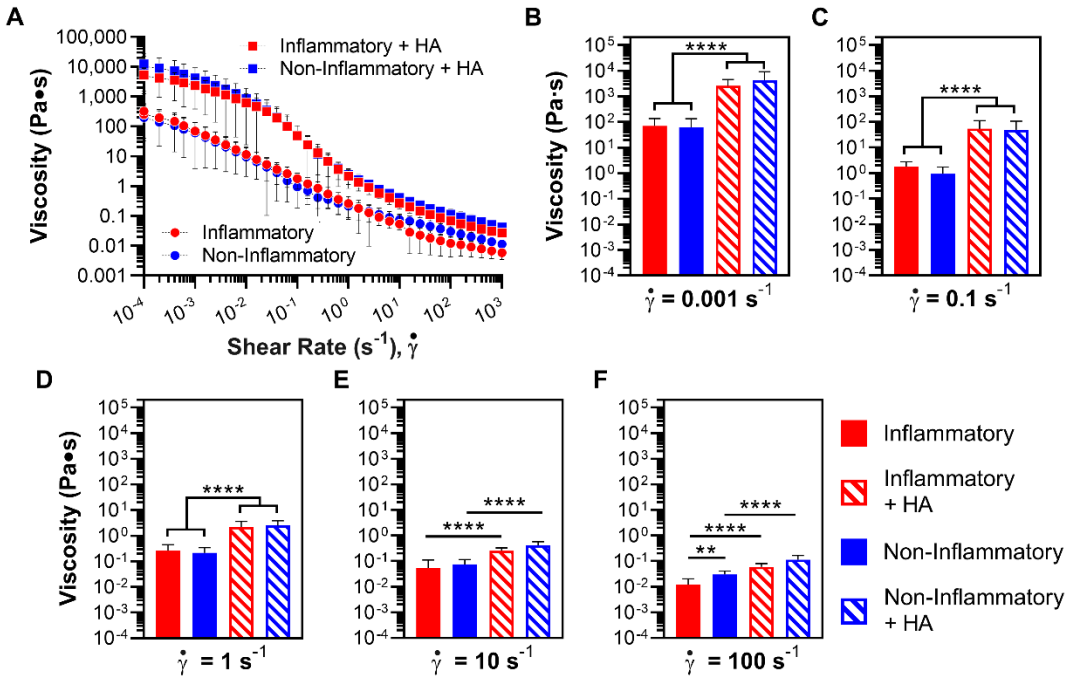

Figure 3-1: (A) Viscosity of inflammatory and non-inflammatory synovial fluids with (■) and without (●) the addition of HA as a function of shear rate, $\dot{\gamma}$. The addition of HA significantly increases mean viscosities of synovial fluids from both non-inflammatory OA and inflammatory arthritis patients at shear rates of 0.001 (B), 0.1 (C), 1 (D), 10 (E), and 100 s^{-1} (F). Asterisks indicate statistically significant differences (*=$p<0.05$, ****=$p<0.0001$, n_{IA}=7-10, n_{IA+HA}=9, n_{OA}=7-9, n_{OA+HA}=8-9).

Further analysis of the fluids' frictional properties was performed using Stribeck analysis, which revealed interesting tribological behaviors. Typical tribological behavior manifested as Stribeck curves exhibiting a sigmoidal shape in which friction decreases with increasing sliding speed, viscosity, or decreasing normal load (Figure 3-2A) [44,54]. This curve is typically broken into three different lubrication modes: boundary, mixed, and elastoviscous modes of lubrication. In the non-inflammatory synovial fluids, friction followed a characteristic Stribeck pattern with and without HA (Figure 3-2B, n_{OA}=7, n_{OA+HA}=7). Friction coefficients exhibited maximal values

at low Sommerfeld numbers of approximately 0.10±0.02 on average, and they decreased with increasing Sommerfeld number to a minimum plateau at 0.06±0.01. The addition of HA resulted in reduced friction coefficients and a shift in the Stribeck curve toward higher Sommerfeld numbers as expected due to the high viscosity of HA. In contrast, inflammatory OA samples showed inconsistent tribological behavior (Figure 3-2C,D). Several exhibited a magnitude of friction coefficients and typical tribological behavior similar to the non-inflammatory OA synovial fluid ($n_{IA,typical}=4$) with friction coefficients below 0.10. However, a subset showed a vastly different behavior we defined as "aberrant" tribological behavior ($n_{IA,aberrant}=4$). Aberrant tribology was defined by friction coefficients that *increased* with Sommerfeld number and/or exhibited more variable friction coefficients. Even with the addition of HA, aberrant tribological behavior was still observed (Figure 3-2D, $n_{IA,typical+HA}=3$, $n_{IA,aberrant+HA}=5$). When quantified by taking the difference between friction coefficients from 0.1 to 10mm/s sliding speed ($\Delta\mu$, Figure 3-2E), only inflammatory synovial fluids exhibited a negative $\Delta\mu$ (Figure 3-2F). The mean value of $\Delta\mu$ was higher in non-inflammatory OA synovial fluids as compared to inflammatory arthritis fluids ($p=0.005$, $n_{OA}=8$, $n_{IA}=8$).

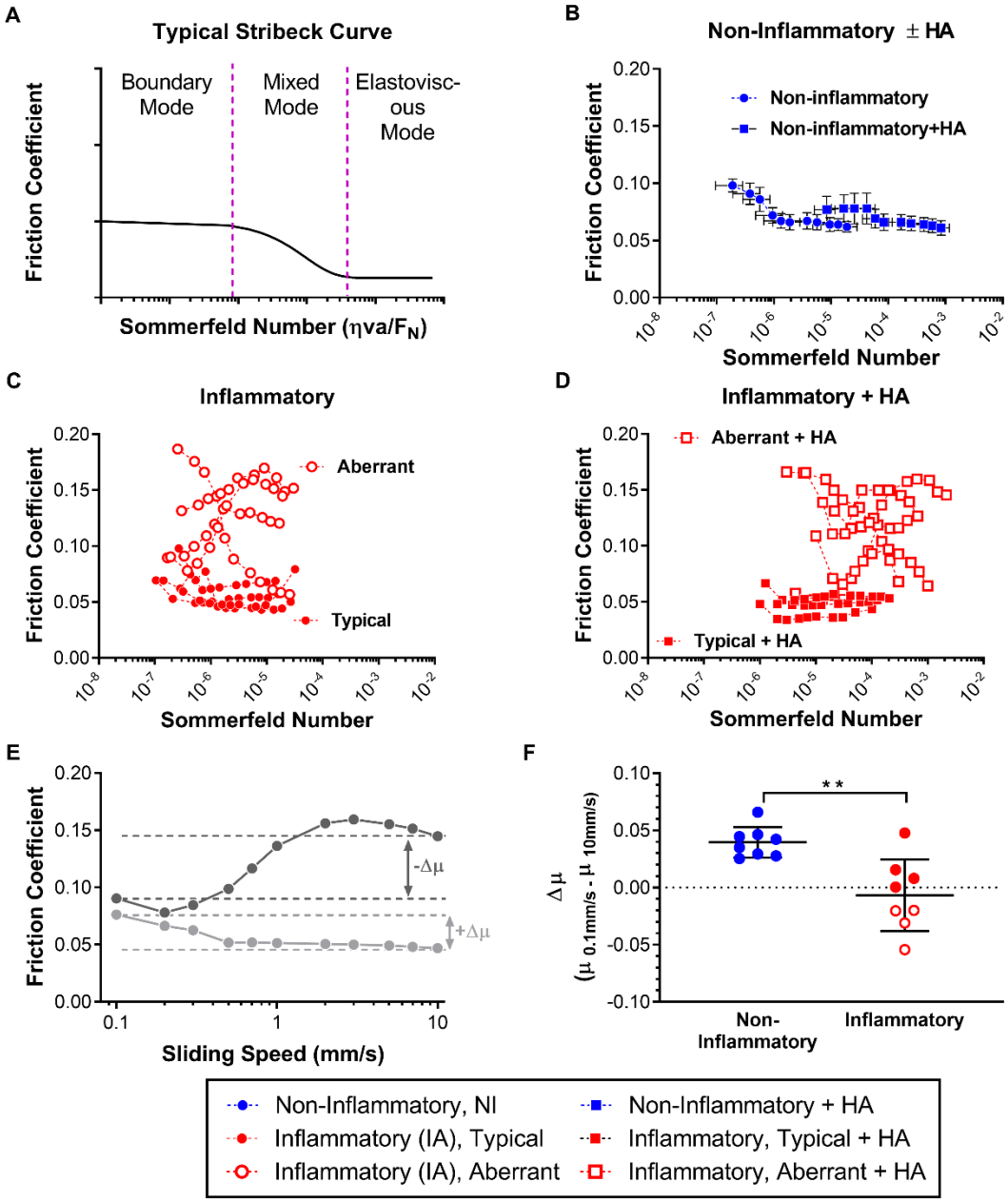

Figure 3-2 : (A) Stribeck curves follow a characteristic sigmoidal shape of decreasing friction coefficient with increasing Sommerfeld numbers. (B) The mean friction coefficients of all non-inflammatory synovial fluids as a function of Sommerfeld number are shown alone (●,n_{OA}=7) and with HA (■,n_{OA+HA}=7). The friction coefficients of each inflammatory arthritis patient's synovial fluid are shown without HA (C, $n_{IA,typical}$=4 , $n_{IA,aberrant}$=4) and with HA (D, $n_{IA,typical+HA}$=3

, $n_{IA,aberrant+HA}$=5). In both (C) and (D), a subset of synovial fluids exhibits typical Stribeck behavior (●,■) while another subset exhibits aberrant tribological behavior (○,□) in which friction increases with Sommerfeld number. For each SF sample, tribological behavior was quantified by calculating the difference in friction coefficient between the slowest and fastest sliding speeds, Δμ, as calculated as illustrated in (E). (F) Only a subset of inflammatory arthritis synovial fluids exhibited aberrant behavior, i.e., a negative Δμ (○), whereas non-inflammatory and inflammatory arthritis fluids with typical tribological behavior exhibit positive values of Δμ (*p=0.016, $n_{IA,typical}$=8 , $n_{IA,aberrant}$=8).

Synovial fluids clustered by disease and lubrication endotype along the model Stribeck curve. Inflammatory fluids with aberrant tribological behavior exhibited the lowest effective viscosities of 0.16±0.11 Pa·s (Figure 3-3A, Mean±SEM, $n_{IA,aberrant}$=3, Supplemental Table 3S- 3 and Table 3S- 4). In contrast, the effective viscosities of non-inflammatory fluids and inflammatory fluids with typical tribological behavior were approximately 50 and 150 times greater on average, respectively (n_{OA}=8, $n_{IA,typical}$=5). The addition of HA resulted in approximately a two-fold increase in effective viscosities for non-inflammatory fluids and inflammatory fluids with typical tribological behavior (n_{OA+HA}=7, $n_{IA,typical+HA}$=4). For aberrant fluids, there was a 57-fold increase in mean effective viscosity, but there was a high degree of variation (Figure 3-3A, $n_{IA,aberrant+HA}$=5). Overall, HA did not significantly affect effective viscosities (p=0.54).

When measured viscosities were used to generate Stribeck curves, data among the different endotypes overlapped and did not show a single distinct trend (Figure 3-3B). However, when effective viscosities were used (Figure 3-3C,D, n_{OA}=8, n_{OA+HA}=7, $n_{IA,typical}$=5, $n_{IA,typical+HA}$=4), non-inflammatory and inflammatory synovial fluids with typical tribological behavior clustered in the mixed and elastoviscous regions of the Stribeck curve. Non-inflammatory fluids aligned closely

with the model Stribeck curve with mean RMS errors of 19.6±2.6% and 21.4±3.1% without or with HA, respectively (Mean±SEM, Supplemental Table 3S- 4). Inflammatory fluids with typical tribological behavior also appeared to align closely with the model Stribeck curve, but they exhibited elevated mean RMS errors of 23.9±3.7% and 19±3.7% without or with HA, respectively. In contrast, inflammatory fluids with aberrant tribological behavior clustered in the boundary mode and early phase of the mixed lubrication mode (Figure 3-3D, $n_{IA,aberrant}=3$, $n_{IA,aberrant+HA}=5$). They also exhibited a higher degree of variation from the model Stribeck curve as compared to fluids with typical tribological behavior with mean RMS errors of 27.1±2.2% and 27.3±9.8% without and with HA, respectively.

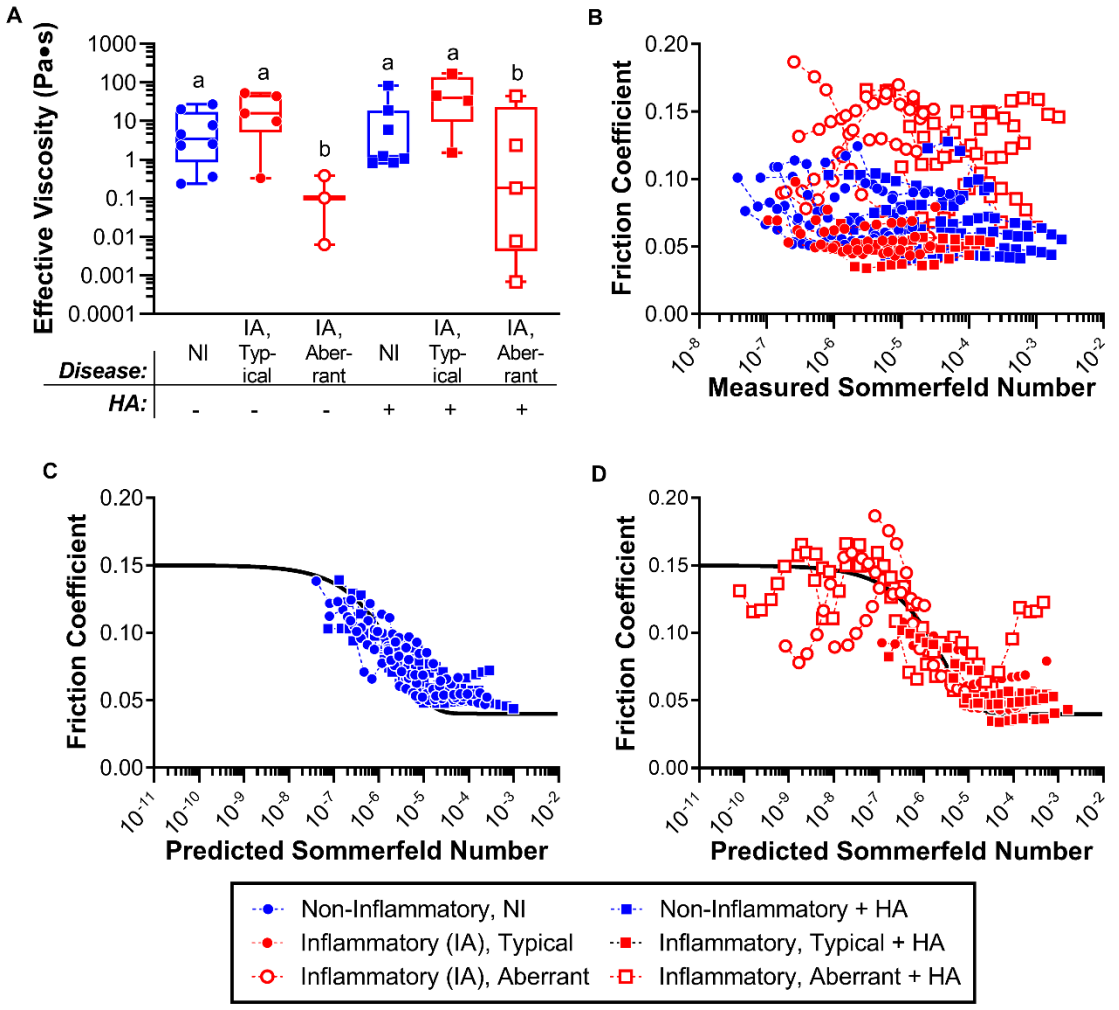

Figure 3-3: (A) Inflammatory arthritis (IA) synovial fluids with aberrant tribological behavior exhibit lower effective viscosities than non-inflammatory (NI) OA and IA synovial fluids with typical tribological behavior (n_{OA}=8, $n_{IA,typical}$=5, $n_{IA,aberrant}$=3, n_{OA+HA}=7, $n_{IA,typical+HA}$=4, $n_{IA,aberrant+HA}$=5). Differing letters denote significant differences. (B) Stribeck curves using measured viscosities overlap and do not exhibit a unified Stribeck pattern. (C) NI synovial fluids (n_{OA}=8, n_{OA+HA}=7) and (D) IA synovial fluids with typical tribological behavior ($n_{IA,typical}$=5, $n_{IA,typical+HA}$=4) collapsed well onto a model Stribeck curve when effective viscosities were used to calculate Sommerfeld number, and they were concentrated in the mixed and elastoviscous lubrication modes. (D) IA synovial fluids with aberrant tribological behavior ($n_{IA,aberrant}$=3,

$n_{IA,aberrant+HA}$=5) were concentrated in the boundary and early mixed modes of the Stribeck curve, and they tended to deviate more from the model Stribeck curve. Note that individual patient samples are plotted with data points from the same sample connected by a dashed line.

With respect to lubricant composition, inflammatory fluids with aberrant tribology were characterized by low concentrations of lubricin. In both non-inflammatory and inflammatory fluids with typical tribological behavior, lubricin concentrations were higher and more variable, though a significant difference was only detected between the two types of inflammatory fluids (Figure 3-4A, p=0.021, n_{OA}=10, $n_{IA,typical}$=6, $n_{IA,aberrant}$=4). The concentration of HA was elevated in non-inflammatory synovial fluids as compared to inflammatory fluids with typical tribological behavior (Figure 3-4B, p=0.005, n_{OA}=10, $n_{IA,typical}$=6, $n_{IA,aberrant}$=4). Boundary mode friction coefficients (collected at 0.1mm/s sliding speed) decreased with increasing lubricin concentrations (Figure 3-4C) and these data moderately followed a one-phase decay model (R^2=0.36, n=17, p=0.16). There was no evidence of a trend between friction coefficient and HA concentration (Figure 3-4D, R^2=0.03, n=17, p=0.38).

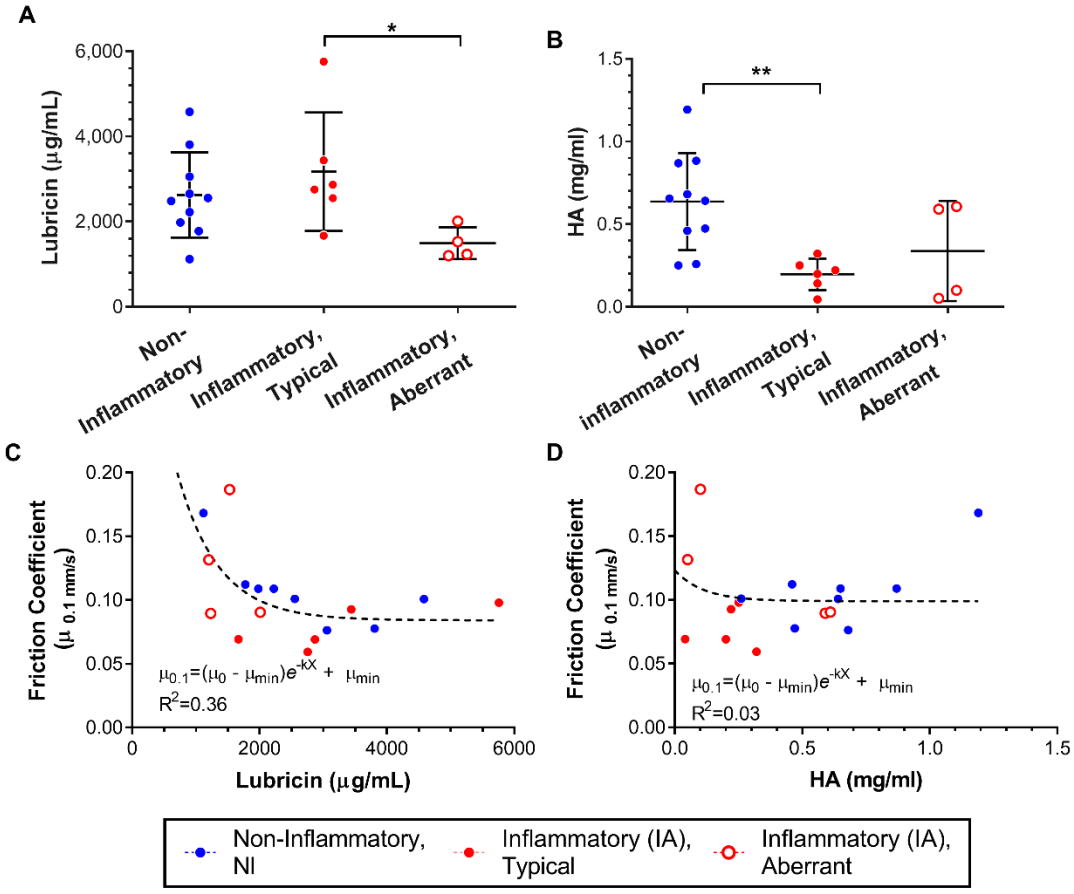

Figure 3-4: (A) Inflammatory arthritis fluids with aberrant tribological behavior exhibited reduced lubricin concentrations compared to fluids with typical tribological behavior (n_{OA}=10, $n_{IA,typical}$=6, $n_{IA,aberrant}$=4). (B) Hyaluronic acid concentrations were greater in non-inflammatory synovial fluids compared to inflammatory fluids with typical tribological behavior (p=0.01, n_{OA}=10, $n_{IA,typical}$=6, $n_{IA,aberrant}$=4). (C) Friction coefficients decreased with increasing lubricin concentrations (one-phase decay model, R^2=0.36, n=17, p=0.16). Inflammatory samples with aberrant tribological behavior tended to have low lubricin concentrations in contrast to inflammatory fluids with typical tribological behavior or non-inflammatory synovial fluids. (D) Friction coefficients were not explained by HA concentrations (R^2=0.03, n=17, p=0.38).

3.5 Discussion

The goal of this study was to determine differences in synovial fluid lubrication between inflammatory arthritis and non-inflammatory OA synovial fluids and how supplementation with HA differentially affected lubrication in these cohorts. HA significantly increased the viscosity of synovial fluids from both cohorts, but the viscosities of synovial fluids alone were similar between cohorts. Non-inflammatory OA fluids and some of the inflammatory arthritis fluids exhibited typical tribological behavior with friction coefficients that decreased with Sommerfeld number and were less than approximately 0.10 in magnitude. HA effectively lowered friction coefficients in non-inflammatory OA fluids by approximately 0.02 at the lowest sliding speeds. However, the Stribeck curves for another subset of the inflammatory arthritis fluids were quite different; in this subgroup which we defined as exhibiting aberrant tribological behavior, friction coefficients exceeded approximately 0.10 in magnitude and *increased* with Sommerfeld number. Interestingly, HA failed to eliminate the aberrant tribological behavior in inflammatory arthritis fluids; high friction coefficients that increased with Sommerfeld number were still observed after the addition of HA. When friction data were mapped onto a model Stribeck curve, aberrant fluids exhibited a reduced effective viscosity and clustered in the boundary and mixed modes of lubrication. Furthermore, HA did not significantly increase the effective viscosity, which contrasts against viscosity measurements made on the rheometer. Relatively low lubricin concentrations may distinguish aberrant synovial fluids from fluids with typical tribological behavior.

The absence of difference in measured viscosity between inflammatory and non-inflammatory synovial fluids was surprising. Previous studies on the steady shear viscosity of synovial fluids have generally found that low shear rate or zero-shear viscosities are lower in osteoarthritis by approximately 1-2 orders of magnitude and lower still in inflammatory joint

pathologies like rheumatoid arthritis or monoarthritis by approximately 2-3 orders of magnitude [63–65] as compared to post-mortem or healthy control fluids. The magnitude of viscosities for diseased samples in this study at low shear rates were on the order of 100 Pa·s, which is much greater than previous studies reporting viscosities ranging from 0.1-5 Pa·s for OA and 0.004-0.7 for inflammatory arthritis [64,65]. The viscosities measured here more closely parallel those reported for healthy synovial fluids [66]. The disparity may be explained by differences in disease severity and how viscosity was measured. We and others have both used Couette-type geometries including cone-on-plate geometries [64] and coaxial cylinder viscometers [65]. In model synovial fluid solutions, the magnitude of zero-shear viscosity measurements was demonstrated to vary with geometry due to interfacial effects [67], and thus differences in geometry, material, and size may drive variation in viscosity magnitudes across studies. Notably, in the present study, HA concentrations and average joint fluid volumes (Supplemental Table 3S- 1) were similar between non-inflammatory OA and inflammatory arthritis fluids, and therefore similar viscosities would also be expected. The variation in synovial fluid mechanics supports the growing consensus that OA is a highly variable disease.

As revealed by application of the Stribeck framework, the tribological behavior of synovial fluids from patients with inflammatory arthropathies was highly variable and unique as compared to other lubricants. To our knowledge, this is the first time that the Stribeck framework has been applied to diseased synovial fluid. Previous studies investigating lubricants' multi-modal, cartilage friction properties have not shown an increase in friction coefficient with an increase in Sommerfeld number, or more specifically to this study, with an increase in sliding speed [44,68–70]. Aberrant tribological behavior was observed only in inflammatory fluids; friction coefficients were higher, more variable, and increased with Sommerfeld number as compared to fluids with

typical tribology. Importantly, adding HA differentially affected lubrication across patients; a lowering of friction coefficients occurred in non-inflammatory and some inflammatory samples, but it did not eliminate the aberrant, high friction behavior. OA is recognized as a collection of diseases with differing endotypes, and defining them is an active area of research [27,71]. Previous research has suggested defining arthritis endotypes based on synovial fluid viscosity. Our results suggest that frictional properties may also distinguish different forms of arthritis and be a useful indicator, especially for inflammatory arthropathies, which exhibited highly variable tribological behavior. Furthermore, baseline synovial fluid friction behavior may influence how effective an HA viscosupplement or other lubrication therapy may be to treating arthritis. Future studies are needed to fully understand what varieties of lubrication-based endotypes exist and what mechanisms drive them.

Synovial fluids were further differentiated by their effective viscosities and subsequently, position on a model Stribeck curve. Given that there were no differences in measured viscosity, the Stribeck curves for the different lubricants overlapped and did not form a continuous Stribeck curve. In a recent publication, friction data on commercial viscosupplements exhibited a similar behavior, but when effective viscosities were used, the data collapsed well onto a model Stribeck curve for dextran, a linear polymer [23]. Furthermore, the effective viscosities correlated strongly with friction coefficient. Here, the same technique was employed, though the model Stribeck curve's boundary mode friction coefficient was reduced to account for synovial fluids' generally lower friction coefficients compared to dextran, which is likely due to additional lubricating molecules like lubricin present in synovial fluid. The resulting Stribeck curves showed a clear separation of the inflammatory fluids with aberrant tribological behavior to the boundary and

mixed mode lubrication regimes of the model Stribeck curve, whereas fluids with typical tribological behavior were concentrated in the mixed and elastoviscous modes of lubrication.

Importantly, the aberrant fluids with high friction coefficients and low effective viscosities were characterized by low lubricin concentrations. These results are consistent with previous studies that have shown that lubricin is a critical player in viscous lubrication because it facilitates HA surface adsorption, which, in turn, increases viscosity at the cartilage surface [54,72,73]. This aggregation may also explain why aberrant tribological behavior persisted even after HA supplementation. Lubricin has also been found to dramatically affect the magnitude of friction and the shape of the Stribeck curve [54]. Low synovial lubricin concentrations have been found in a variety of arthritic conditions and species, including ligament tears in rodents [74,75], guinea pigs [76], rabbits [2], and humans [38], and human OA and rheumatoid arthritis [7]. In small [75,77–81] and large animal models [82] of OA, therapeutic intra-articular delivery of lubricin and polymeric lubricin-mimetics [83] has been demonstrated to improve cartilage morphology [75,77–80,82,83], reduce inflammation [82] and cartilage breakdown products [77,79,81,82], and improve gait [77]. In addition to lubricin, aberrant fluids also had high levels of IL-8 (Supplemental Table 3S- 2 and Supplemental Figure 3S- 2). IL-8 is a chemokine known to produce pro-inflammatory effects in the joint such as recruitment of neutrophils, which produce neutrophil elastases capable of degrading lubricin [84–87]. While it is well known that HA and lubricin function synergistically to lubricate cartilage, our results suggest that reduced lubricin concentrations may encumber an HA viscosupplement's lubricating potency.

Though rotational rheometry demonstrated 18- to 90-fold increases in viscosity with the addition of HA, the effective viscosities of fluids with and without HA were not different. Effective viscosities varied across six orders of magnitude and were right-skewed with a median of 2.3 Pa·s,

which is similar to viscosities collected on the rotational rheometer in this study at shear rates of 0.1-1s^{-1}. In this study, viscosupplementation did not substantially reduce friction. Since the process of mapping data onto a model curve is governed by minimizing error between measured and predicted friction coefficients, it is reasonable that similar friction coefficients would lead to similar effective viscosities. For non-inflammatory fluids and inflammatory fluids with typical tribological behavior, friction coefficients without HA were low and reached a minimum friction plateau equivalent to fluids with HA. Therefore, the lack of difference in effective viscosity may be explained by a saturation in lubrication. In contrast, aberrant fluids exhibited high friction and a consequent large potential for lubrication. However, friction coefficients with and without HA were similar for the aberrant fluids. Phenomenologically, the lack of difference in effective viscosities between aberrant fluids with and without HA suggests that something in or absent from the fluids limits HA's lubricating ability. For example, low levels of lubricin in these fluids may have limited HA adsorption to the cartilage surface and efficient lubrication [54,72,73]. Traditional rheology techniques, like rotational rheometry, may not accurately capture physiologic synovial lubrication or correlate well with frictional properties [23]. For example, these techniques do not incorporate cartilage surface interactions. Through use of a surface force apparatus, surface interactions between lubricin, HA, and albumin have been found to affect friction and viscosity [73,88]. Furthermore, steady shear rheology captures only the shear thinning behavior of synovial fluid at a constant gap distance, which may not accurately mimic the viscous behavior in a joint or in our linear, cartilage-on-glass tribometer setup. Altogether, these results motivate the need to develop more advanced rheometry methods that overcome such limitations.

Several limitations exist for this study. A relatively small cohort size was evaluated. Due to limited volumes of synovial fluid, it was not possible to assess all patient samples in both the

tribometer and rheometer as well as with and without HA. Aberrant tribology was only observed in four of the twenty patients, and therefore made it challenging to determine their distinguishing characteristics, such as lubricin concentrations, with high confidence. Still, the high-friction behavior in inflammatory fluids both alone and with HA that characterizes the aberrant endotype motivate the need to fully characterize this tribological endotype. Furthermore, this study focuses only on the effect of HA on synovial fluid mechanics and not biological activity of HA. It is unclear how treatment with HA would affect mechanics in the long-term and how this may differ between non-inflammatory OA and inflammatory arthritis. Previous studies have directly tied changes in lubrication to changes in cartilage health including apoptosis and mitochondrial dysfunction [3–6], and so understanding the mechanics of diseased synovial fluid and their differential responses to an HA viscosupplement are valuable. Finally, HA molecular weight distribution in the synovial fluids was not quantified due to limitations in the volume of synovial fluid. A shift in the distribution could affect the performance of exogenous HA or play a role in aberrant tribological behavior. The similarities in measured viscosities between inflammatory arthritis and non-inflammatory OA suggest that the distributions were likely similar.

Collectively, the results of the present study indicate that frictional properties of synovial fluid can distinguish forms of arthritis. We identified a subset of synovial fluids with aberrant tribological behavior unique to patients with inflammatory arthritis, which exhibited high and variable friction coefficients with an atypical pattern. The effectiveness of an HA viscosupplement in improving synovial fluid mechanics varied with endotype, and it did not eliminate the aberrant tribological behavior. While viscosities measured on a rotational rheometer did not differ between non-inflammatory OA and inflammatory arthritis, samples with aberrant tribology showed markedly lower *effective* viscosities. Furthermore, Stribeck analysis using effective viscosities

demonstrated that aberrant fluids operated in mostly the boundary friction regime as opposed to fluids with typical tribology, which were mostly concentrated in the low-friction modes, i.e., mixed and elastoviscous modes. Aberrant tribology was associated with low lubricin concentrations. Considering the role of lubricin in localizing HA to the cartilage surface, we hypothesize that relatively low lubricin drove the aberrant tribology and likewise limited the effectiveness of exogenous HA. Ultimately, this work suggests that there exist tribological endotypes of arthritis, which may respond differentially to friction-based therapies.

3.6 Acknowledgments

We thank Anton-Paar for providing the MCR-702 rheometer via the Cohen Laboratory as well as the Reesink Laboratory for assistance with the ELISAs. Research reported in this publication was supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-1650441 (EF) and Fidia Farmaceutici.

3.7 Supplemental Figures and Tables

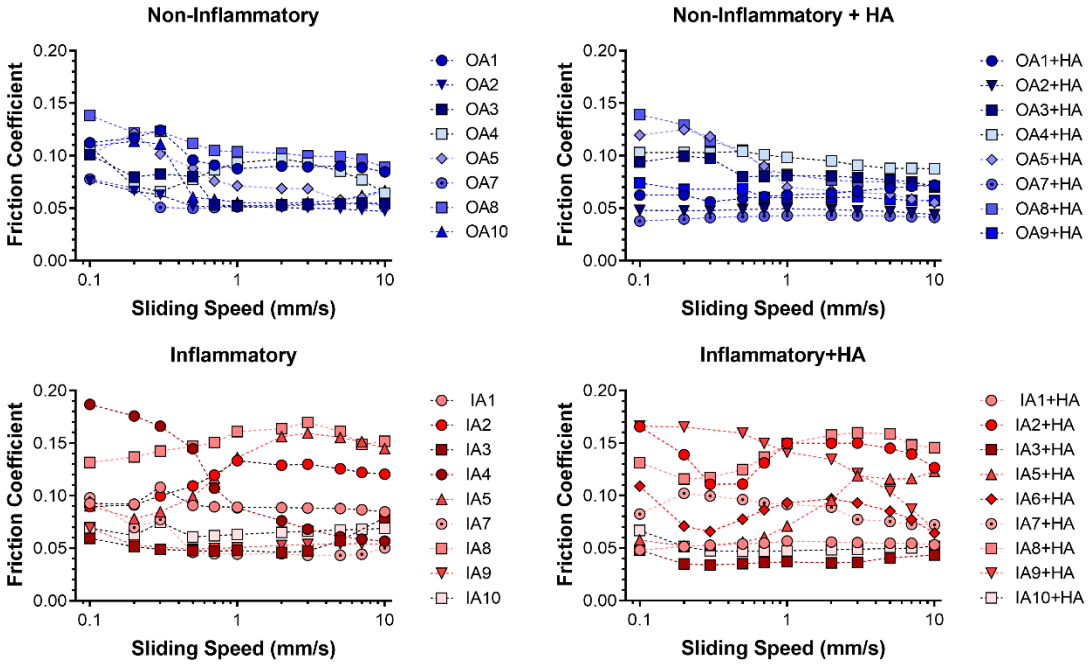

Figure 3S- 1 : Plots of synovial fluid friction versus sliding speed for non-inflammatory OA±HA and inflammatory arthritis±HA are shown. Each line within a plot represents a single patient's friction curve. Patient labels, i.e., OA# or IA#, are consistent with labels in the Supplemental Tables.

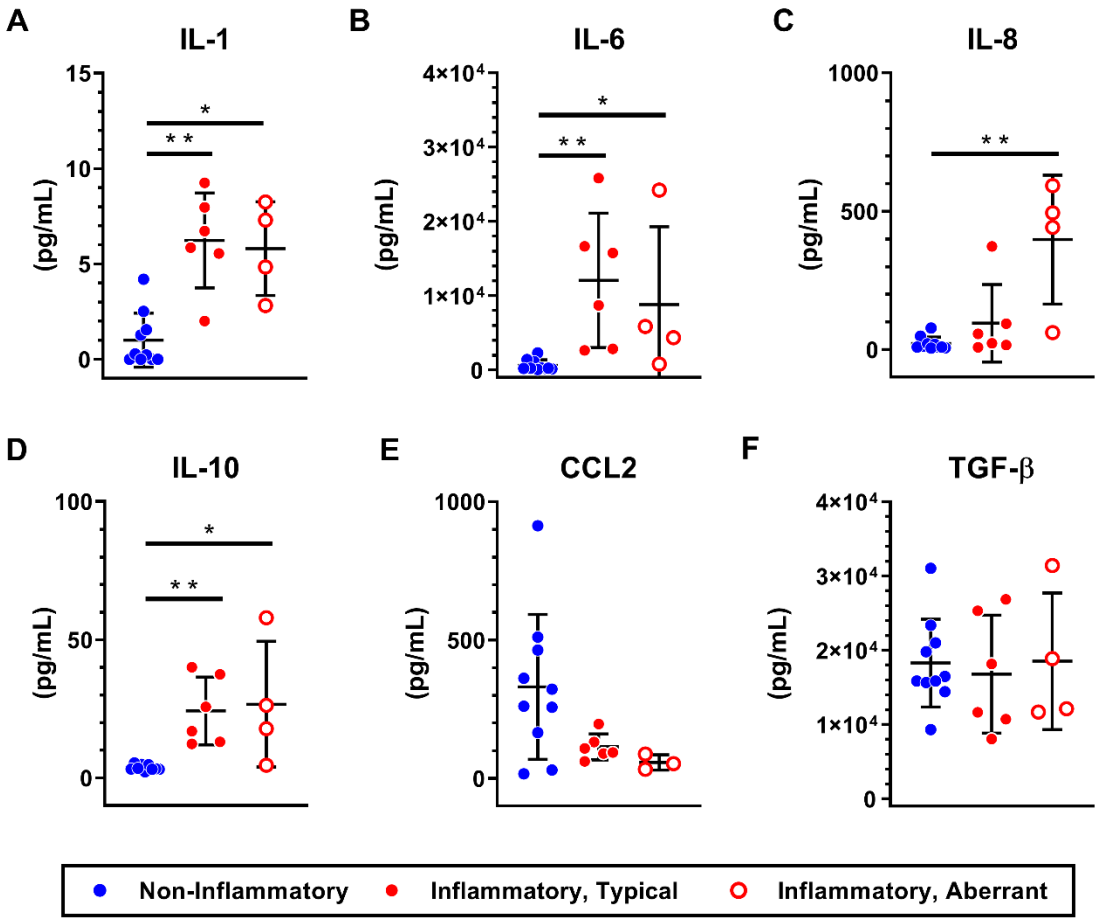

Figure 3S- 2: Cytokine concentrations varied with arthritis endotype with inflammatory synovial fluids exhibiting higher synovial concentrations of IL-1, IL-6, IL-8, and IL-10 than non-inflammatory synovial fluids (A-D, Supplemental Table 3S- 2 and Table 3S- 5). For CCL2 ($p=0.28$) and TGF-β ($p=0.8$), no significant differences were detected among groups

Table 3S- 1 : The synovial fluid cell composition and other patient information are shown. WBC=white blood cell count, PMN%=polymorphonuclear neutrophil percentage, 1st Pres.=1st Presentation, F=female, M=male

Table 3S-1

Non-inflammatory OA

Patient Sample	Flare/1st Pres.	Volume (ml)	Age	Sex	WBC (#/mm^3)	PMN %
OA1	1st Pres.	32	61	F	300	-
OA2	1st Pres.	60	63	F	200	4
OA3	Flare	25	60	F	200	-
OA4	Flare	160	70	F	300	-
OA5	1st Pres.	20	48	M	100	-
OA6	Flare	50	73	F	100	-
OA7	Flare	20	72	F	100	-
OA8	Flare	15	55	F	100	-
OA9	1st Pres.	20	48	M	400	-
OA10	Flare	34	72	F	100	1
Mean		43.6	62.2		190	2.5
SEM		4.36	6.22		19	1.25
Minimum		15	48		100	1
Maximum		160	73		400	4

Inflammatory Arthritis

Patient Sample	Disease	Flare/1st Pres.	Volume (ml)	Age	Sex	WBC (#/mm^3)	PMN %
IA1	Crystal-induced arthritis	Flare	18	76	F	9800	84
IA2	Crystal-induced arthritis	Flare	19	90	F	29200	98
IA3	Early Arthritis	1st Pres.	4	21	F	3500	2
IA4	Early Arthritis	1st Pres.	30	27	F	7000	38
IA5	Psoriatic arthritis	Flare	90	65	M	6800	44
IA6	Psoriatic arthritis	1st Pres.	25	23	M	7000	86
IA7	Reactive Arthritis	1st Pres.	18	26	F	7000	22
IA8	Reactive Arthritis	1st Pres.	30		F	4000	64
IA9	Reactive Arthritis	Flare	70	45	F	6800	56
IA10	Spondylo arthritis	Flare	70	28	M	14600	84
Mean			37.4	44.6		9570	57.8
SEM			3.74	4.95		957	5.78
Minimum			4	21		3500	2
Maximum			90	90		29200	98

Table 3S- 2 : The biochemical composition of synovial fluid from each patient is shown. Each row represents an individual patient's synovial fluid analysis data with labels OA# or IA # to represent whether the fluids were classified as non-inflammatory OA or inflammatory arthritis (IA). Patient labels, i.e., OA# or IA#, are consistent with labels in other Supplemental Figures and Tables.

Table 3S-2:

Biochemical Composition

	Patient Sample	IL-1 (pg/ml)	CCL2 (pg/ml)	IL-8 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TGF-β (ng/ml)	HA (mg/ml)	Lubricin (µg/ml)	DISEASE
Inflammatory Arthritis	IA1	9.25	108.1	373.3	16,644.0	25.7	25.3	0.22	3,438.8	Crystal-induced arthritis
	IA2 (Aberrant)	8.24	87.5	441.6	24,220.0	26.3	18.9	0.59	1,231.9	Crystal-induced arthritis
	IA3	7.30	52.8	61.2	4,377.0	17.8	11.7	0.32	2,754.1	Early Arthritis
	IA4 (Aberrant)	6.72	89.7	93.4	15,776.0	40.0	11.6	0.10	1,530.3	Early Arthritis
	IA5 (Aberrant)	2.81	32.9	592.8	800.0	4.5	12.1	0.61	2,009.1	Psoriatic arthritis
	IA6	2.00	93.5	22.3	8,694.0	12.2	10.7	0.14	2,550.9	Psoriatic arthritis
	IA7	5.85	61.0	7.7	2,846.0	16.8	18.2	0.25	5,757.4	Reactive Arthritis
	IA8 (Aberrant)	4.84	1351.8	493.8	5,877.0	58.0	31.4	0.05	1,201.8	Reactive Arthritis
	IA9	5.54	130.8	56.6	2,644.0	37.5	26.9	0.04	1,667.4	Reactive Arthritis
	IA10	7.96	196.6	16.4	25,838.0	13.0	8.1	0.20	2,868.4	Spondyloarthritis
	Mean	6.1	220.5	215.9	10,771.6	25.2	17.5	0.3	2,501.0	
	Std. Dev.	2.3	400.1	230.9	9,218.7	16.0	8.0	0.2	1,368.8	

Table 3S-2:Biochemical Composition

	Patient Sample	IL-1 (pg/ml)	CCL2 (pg/ml)	IL-8 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TGF-β (ng/ml)	HA (mg/ml)	Lubricin (µg/ml)
Non-inflammatory Osteoarthritis	OA1	4.19	164.8	48.2	1,135.4	5.4	23.4	0.46	1,777.7
	OA2	0.00	464.0	11.6	2,306.4	4.9	19.8	0.68	3,058.3
	OA3	0.27	510.8	5.1	249.2	3.1	14.4	0.26	2,555.1
	OA4	2.51	15.9	19.4	134.7	4.7	15.9	0.65	2,223.6
	OA5	0.00	322.6	20.8	233.9	3.3	9.3	0.64	4,579.6
	OA6	0.00	257.4	8.5	227.1	3.5	21.0	0.25	2,484.7
	OA7	1.55	30.0	8.6	1,414.5	11.5	15.7	0.47	3,807.0
	OA8	0.00	912.7	77.5	87.0	2.2	31.0	1.19	1,117.3
	OA9	0.22	260.9	9.0	290.6	3.1	15.9	0.88	2,654.5
	OA10	1.27	361.8	6.0	206.0	3.2	16.5	0.87	1,980.2
	Mean	1.0	330.1	21.5	628.5	4.5	18.3	0.6	2,623.8
	Std. Dev.	1.4	261.9	23.5	743.9	2.6	5.9	0.3	999.9

Table 3S- 3: The RMS Error (%) and the effective viscosity (Pa·s) for each patient sample are shown and ordered by disease type, friction behavior, and presence of HA.

Table3S-3				
HA	**Disease**	**Sample**	**Effective Viscosity**	**RMS Error (%)**
+HA	Inflammatory	IA1	33.54	24.7
		IA3	167.72	10.9
		IA7	1.52	25.8
		IA10	45.93	14.5
	Inflammatory-Aberrant	IA2	0.01	12.9
		IA5	44.65	61.8
		IA6	2.34	36.6
		IA8	0.00	13.0
		IA9	0.18	12.4
	Non-Inflammatory	OA1	19.02	34.2
		OA2	83.10	14.8
		OA3	0.84	26.6
		OA4	0.81	22.9
		OA5	1.23	11.3
		OA8	1.06	14.0
		OA9	5.95	26.1
-HA	Inflammatory	IA1	0.33	28.9
		IA3	52.98	27.2
		IA7	15.76	11.7
		IA9	44.20	19.8
		IA10	9.98	32.0
	Inflammatory-Aberrant	IA2	0.10	26.0
		IA4	0.38	23.9
		IA5	0.01	31.3
		IA8	*Solution did not converge*	
	Non-Inflammatory	OA1	0.24	20.8
		OA2	20.21	13.6
		OA3	7.77	15.9
		OA4	2.32	36.6
		OA5	2.54	17.6
		OA7	27.08	17.8
		OA8	0.36	13.4
		OA10	4.52	21.4

Table 3S- 4 : Summary statistics of the effective viscosity (Pa·s) and RMS error (%) are shown for each group of lubricants.

Table 3S- 4: Summary Statistics on Viscosity and RMS Error for Theoretical Stribeck Curve Fits

HA	Disease		Mean	Std. Dev.	Median	Minimum	Maximum	n	SEM
- HA	Inflammatory	Effective Viscosity	24.7	22.8	15.8	0.3	53.0	5	10.2
		RMS Error (%)	23.9	8.2	27.2	11.7	32.0	5	3.7
	Inflammatory-Aberrant	Effective Viscosity	0.2	0.2	0.1	0.0	0.4	3	0.1
		RMS Error (%)	27.1	3.8	26.0	23.9	31.3	3	2.2
	Non-Inflammatory	Effective Viscosity	8.1	10.0	3.5	0.2	27.1	8	3.6
		RMS Error (%)	19.6	7.5	17.7	13.4	36.6	8	2.6
+ HA	Inflammatory	Effective Viscosity	62.2	72.8	39.7	1.5	167.7	4	36.4
		RMS Error (%)	19.0	7.4	19.6	10.9	25.8	4	3.7
	Inflammatory-Aberrant	Effective Viscosity	9.4	19.7	0.2	0.0	44.6	5	8.8
		RMS Error (%)	27.3	21.9	13.0	12.4	61.8	5	9.8
	Non-Inflammatory	Effective Viscosity	16.0	30.3	1.2	0.8	83.1	7	11.5
		RMS Error (%)	21.4	8.3	22.9	11.3	34.2	7	3.1

Table 3S- 5: Table of statistical test results organized by parameter analyzed. KW=Kruskal Wallis Test by Ranks.

Table 3S-5

Parameter	Test	Chi Squared, $\chi 2$	df	p-value	Post-Hoc Comparison: p-values OA-IA	OA-IA (Aberrant)	IA-IA (Aberrant)
Lubricin	KW	6.29	2	0.043	0.6	0.076	0.021
Hyaluronic Acid	KW	9.57	2	0.008	0.005	0.095	0.77
IL-1	KW	12.73	2	0.002	0.003	0.013	1
IL-6	KW	12.9	2	0.002	0.001	0.029	0.9
IL-8	KW	9.43	2	0.009	0.25	0.004	0.14
IL-10	KW	12.11	2	0.002	0.003	0.018	1
CCL2	KW	2.52	2	0.28			
TGF-β	KW	0.44	2	0.8			
$\Delta\mu$ ($\mu_{0.1mm/s}$-$\mu_{10mm/s}$)	Welch's t-test		9.2		0.004		

Parameter	Test	Wald Chi Squared, $\chi 2$	df	p-value			
Effective Viscosity	Mixed Model						
	Fixed Effect: Disease	19.6	2	5.50E-05	0.31	0.012	0.0015
	Fixed Effect HA	0.41	1	0.52			
	Interaction Term: Disease*HA	Removed due to non-significance					

					Post-Hoc Comparison: p-values HA- No HA			
Rotational Rheology, Shear Rate=0.001 s^{-1}	Mixed Model							
	Fixed Effect: Disease	0.72	1	0.72				
	Fixed Effect HA	<2E-16	1	4.90E-11	<0.0001			
	Interaction Term: Disease*HA	Removed due to non-significance						
Rotational Rheology, Shear Rate=0.1 s^{-1}	Fixed Effect: Disease	1.16	1	0.3				
	Fixed Effect HA	114	1	2.10E-08	<0.0001			
	Interaction Term: Disease*HA	Removed due to non-significance						
Rotational Rheology, Shear Rate=1 s^{-1}	Fixed Effect: Disease	5.20E-03	1	0.94				
	Fixed Effect HA	183	1	7.50E-11	<0.0001			
	Interaction Term: Disease*HA	Removed due to non-significance						
					IA vs OA	**IA + HA vs OA+ HA**	**IA vs IA +HA**	**OA vs OA + HA**

Table 3S-5								
Rotational Rheology, Shear Rate=10 s^{-1}	Fixed Effect: Disease	4.39E+00	1	0.036	1.90E-01	0.19	<0.0001	<0.0001
	Fixed Effect HA	219.8	1	3.30E-11				
	Interaction Term: Disease*HA	Removed due to non-significance						
Rotational Rheology, Shear Rate=100 s^{-1}	Fixed Effect: Disease	11.6	1	0.0007	0.0016	0.1323	p<0.0001	p<0.0001
	Fixed Effect HA	307.1	1	<2.2E-16				
	Interaction Term: Disease*HA	5.9	1	0.015				

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CHAPTER 4

Temporal Changes in Synovial Fluid Lubrication and Composition Following Anterior Cruciate Ligament Tear Injuries and Reconstruction

4.1 Abstract

While synovial lubrication is essential to joint health, the alterations to synovial fluid (SF) lubrication following anterior cruciate ligament tears (ACLT) and ACL reconstruction (ACLR) remain largely unknown in humans. In this study, the temporal variation in synovial fluid (SF) composition and lubrication were investigated following ACLT and ACLR. Additionally, the effect of in vitro viscosupplementation on lubrication was examined. HA concentrations were low following ACLT but increased to normal levels with time. HA polydispersity was similar at the pre-operative and surgical timepoints but increased significantly after ACLR. Lubricin concentrations were similar among timepoints ($p>0.05$) and tended to decrease between the pre-operative and surgical timepoints. Despite the changes in HA and lubricin in the synovial fluid, friction coefficients did not vary significantly with time. The addition of exogenous HA generally reduced friction coefficients by a mean of -0.03 relative to pure SF. Low speed friction coefficients and effective viscosity measurements revealed that viscosupplementation's effect on lubrication depended on not only SF HA concentrations but also native lubricin concentrations. Importantly, increased concentrations of lubricin were correlated with enhanced lubrication by exogenous HA. Finally, of all the cytokines, growth factors, and enzymes measured, only IL-6, MCP-1, IL-1RA, and MMP-3 exhibited significant variation across timepoints. Pre-operative and surgical concentrations of these factors were similar, but lower compared to post-operative concentrations. Taken together, these results suggest that HA is substantially affected by ACLT and that viscosupplementation therapy may require adequate synovial lubricin to improve lubrication.

4.2 Introduction

Post-traumatic osteoarthritis (PTOA) accounts for 12% of all OA [1]. Compared to people without a history of knee injury, the risk of OA development is estimated to be 10-fold higher for patients that have suffered a ligamentous or meniscal injury [2]. Anterior cruciate ligament tears (ACLT) are common with estimates in the United States ranging from 80,000-250,000 cases per year [3]. Approximately 130,000 surgical reconstructions of the ACL occur each year, but they do not protect against the development of PTOA [4–6]. Despite the large burden of PTOA resulting from ACLT, factors contributing to its pathogenesis remain unclear. Numerous risk factors have been identified including patient demographics (age, sex), genetics [7], cartilage impact energy during the traumatic injury [2], concomitant damage to menisci or cartilage at the time of injury [7], and time between injury and repair surgery [5]. Significant interest exists for the detection of early biomarkers of disease such as pro-inflammatory cytokines, catabolic enzymes, and cartilage breakdown products [8], which could then be targeted with interventional therapies to prevent PTOA progression.

In addition to biological changes, several studies have reported significant changes in the mechanical properties of synovial fluid following traumatic joint injuries. Mechanical changes are tied to fluctuations in the lubricating molecules lubricin and hyaluronic acid (HA). Alterations in lubricant composition are known to drive the frictional properties of synovial fluid with decreases in lubricin resulting in inferior boundary lubrication [9–13] and a loss of HA resulting in reduced synovial fluid viscosity and increased friction coefficients [14–16]. Inferior lubrication promotes articular cartilage degradation and chondrocyte death [11,13,17,18]. Numerous animal studies have examined lubricin and HA changes after traumatic joint injuries. Decreases in lubricin have been found in guinea pigs [10] and rats [19] with induced ACLT. In contrast, lubricin has been

found to increase in several equine studies (cartilage impact injuries [20], osteochondral fragmentation [14,20–22], and full-thickness cartilage defects [15,20]), cranial cruciate ligament tears in canines [23], sheep with induced ACLT [24], and human articular fractures [16]). HA concentrations have been found to decrease and shift toward low molecular weights in PTOA in humans [16] and large animals [14,15,22,24] and vary temporally. Decreases in synovial lubricin and HA have motivated the development and use of lubrication-based therapies including HA viscosupplementation [25,26] or tribosupplementation with lubricin and lubricin mimetics [27–30]. However, the variation in lubrication presents a major obstacle for clinicians treating PTOA and OA in general. To tailor lubrication-based therapies for PTOA to a specific mechanical insult and patient, knowledge of synovial fluid mechanics and lubricant composition are necessary.

Notably, few studies have investigated temporal variations in synovial fluid mechanics and lubricating molecules after ACLT and ACL reconstruction. A seminal study by Elsaid et al. in 2008 [31] reported that synovial lubricin concentrations are reduced after ACLT compared to healthy control fluid, and this is the only study of lubricin concentrations in human synovial fluid following ACLT. Furthermore, only one large animal study on the effect of ACLT on lubrication has been conducted [24]. The results showed that in the early time period following ACLT (2-4 weeks), boundary lubrication was diminished, and this was associated with low concentration of high molecular weight HA. In contrast to Elsaid et al. [31], lubricin concentrations were elevated early after ACLT compared to twenty weeks post-ACLT. Though proper synovial lubrication is essential to joint health, temporal variations in synovial lubrication and composition following ACLT and ACL reconstruction in humans is unknown. The goals of this study were (1) to characterize changes in SF composition (lubricin, hyaluronic acid (HA), HA molecular weight, several cytokines and proteases) and lubrication (cartilage-on-glass friction, SF viscosity) and (2)

to determine how in vitro viscosupplementation affected lubrication over the course of ACLT and ACLR.

4.3 Methods

4.3.1 Synovial fluid collection and storage:

Synovial fluid was sampled from the affected knee joints of patients (n=9) with an anterior cruciate ligament tear (ACLT) during treatment at New York University Langone Orthopedic Hospital (IRB Protocol Number: I15-00929). Patients' ages ranged from 18-38 years with a mean value of 27.1±6.7 years, and of the total 9 patients, 5 were male and 4 were female. Fluid was sampled at three broadly defined time points: post-injury (n=5, 7.8±6.8 days after ACLT, mean±SD), day of reconstruction surgery (n=8, 43.1±17.9 days after ACLT, mean±SD), and, if patients presented with a joint effusion, post-operatively (n=6, 7.8±2.0 days after surgery, mean±SD). The maximal amount of fluid was aspirated using a superolateral approach relative to the patella and an 18-gauge needle. The samples were transferred from the collection syringe to sterile tubes and mixed at a 1:100 dilution with a protease inhibitor cocktail solution (Halt™ Protease Inhibitor Cocktail, EDTA-free 100x; Pierce Biotechnology, Rockford, IL). Samples were stored on ice during transport to a laboratory where they were centrifuged at 2260 x g for 10 minutes. Afterwards, the supernatant was aliquoted into sterile tubes and stored at -80°C.

4.3.2 Compositional analysis

4.3.2.A HA ELISA & Gel Electrophoresis

As described previously [14,32], the concentration of hyaluronic acid was quantified in triplicate using a commercial ELISA kit (HA DuoSet ELISA, R&D Systems, Minneapolis, MN). Synovial fluids were diluted 1:80,000 and 1:100,000 in 5% Tween 20 in PBS, and the results for

the 1:80,000 dilution were chosen for presentation due to their lower intra-assay coefficients of variation of 3.3±1.4%.

To assess the molecular weight distribution of HA in the synovial fluids, gel electrophoresis was performed using methods previously described [14,33,34]. Briefly, synovial fluid samples were diluted 1:20 in PBS and digested overnight in Proteinase K (Proteinase K, recombinant, PCR grade, Roche Applied Science, Mannheim, Germany). Samples were loaded into a 0.5% agarose gel along with HA standards, Select-HA HiLadder (0.5–1.5 MDa) and Mega-HA Ladder (1.5–6.1 MDa; Hyalose, Austin, TX). Electrophoresis was conducted at 56V for 8h, and gels were stained overnight with 0.005% Stains-All (Sigma-Aldrich, St. Louis, MO) in 50% ethanol. The following day, gels were de-stained in a 10% ethanol solution for 48h. Gel images were acquired using a Bio-Rad VersaDoc Imaging System (Hercules, CA). The standards' band intensity and position and each lane's intensity profile were quantified in ImageJ. As described previously [34], a linear regression curve was fit to relate the log of molecular weight to band position. This relationship was then used to assess the molecular weight distribution of the synovial fluids including their weight average molecular weight ($\overline{M}_w$), number average molecular weight ($\overline{M}_n$), and polydispersity index (PDI).

4.3.2.B Lubricin ELISA

The concentration of the boundary lubricant lubricin in the synovial fluid samples was determined via an ELISA as previously described [14,21,32]. Briefly, samples were analyzed in triplicate at dilutions of 1:8,000 and 1:16,000 in PBS. A sandwich ELISA was performed using peanut agglutinin (capture reagent), mAb 9G3 (detection antibody, Millipore Sigma, Burlington, MA), and lubricin purified from bovine synovial fluid as the standard. The 1:8,000 dilution was

chosen for presentation due to lower average intra-assay coefficients of variation of 4.9±3.1% and 3.9±2.0%.

4.3.2.C Cytokine ELISAs

The synovial fluid samples were analyzed to determine the concentration of 12 cytokines and chemokines that have previously been suggested to play a role in cartilage degradation and inflammation in the joint space. These included monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), matrix metalloproteinase-3 (MMP-3), chemokine ligand 5 (RANTES), fibroblast growth factor-2 (FGF-2), tissue inhibitor of metalloproteinases (TIMP-1 and TIMP-2), interleukin-1 receptor antagonist (IL-1Ra), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1 beta (MIP-1β). Custom and standard pre-coated multiplex Human V-PLEX Plus ELISA plates from MSD (Meso Scale Discovery, Rockville, Maryland, USA) were selected. MSD Human V-PLEX Plus Kits employed in this study included: Human MMP-3 Ultra-Sensitive, Human chemokine ligand 5 (RANTES) Ultra-Sensitive, V-PLEX Human bFGF Kit, 96-well 4-Spot Prototype (MSD) Human TIMP-1, TIMP-2, 96-well 4-Spot Prototype (MSD) human interleukin-1 receptor antagonist (IL-1Ra), and MULTI-SPOT® 4 Spot Special Order Human 4plex ANALYTES: IL-6, vascular endothelial growth factor (VEGF), MCP-1, macrophage inflammatory protein-1 beta (MIP-1β). Experiments were performed according to the manufacturer's instructions with minimal modifications and optimization. Synovial fluid samples were assayed in duplicate. Plates were read using a MSD QuickPlex SQ120 Plate Reader/Scanner. During analysis, MSD Discover Workbench 4 software generated a standard curve which was compared to the standard curve provided with each kit, and if the results fell within the range determined by the standard curve, they were accepted.

4.3.3 Lubrication Analysis

The synovial fluids' lubricating properties were assessed using established techniques for tribology [14,35,36] and Stribeck analysis [14,36,37].

Friction coefficients were measured using a custom cartilage-on-glass tribometer as previously described [14,35,36]. Briefly, synovial fluids were loaded into the tribometer wells alone or in 1:1 ratio with Hymovis®, a modified HA viscosupplement [32,38]. Cylindrical cartilage explants were harvested from the femoral condyles of neonatal bovids, loaded into the synovial fluid or synovial fluid-HA baths, and compressed to approximately 30% compressive strain against glass. Cartilage-on-glass friction coefficients are similar to those measured in cartilage-cartilage tribometers [39–41] and have been found to correlate with the clinical efficacy of several viscosupplements [42]. After one hour of depressurization to remove effects of interstitial fluid pressure on lubrication, the glass counterface was reciprocated linearly in a random sequence of speeds ranging from 0.1-10mm/s. Throughout the test, a biaxial load cellrecorded the normal and shear loads. For both the forward and reverse directions and at each speed, the friction coefficient was calculated as the mean shear force at the end of sliding when friction had reached an equilibrium value divided by the mean normal load during sliding. The change in friction with the addition of HA was calculated as the difference in friction coefficients at 0.1mm/s sliding speed between synovial fluids with and without exogenous HA, Hymovis®.

Effective viscosity describes the viscosity of a lubricant that gives rises to its lubricating properties. It has been especially useful for describing shear-thinning fluids and soft contacts where traditional rheological measurements fail to accurately predict lubricating behavior [37,43–45] The effective viscosities of the synovial fluids with and without HA were calculated as previously described using Stribeck analysis [37]. Stribeck analysis is a means of mapping the

relationship between friction coefficient and the operating conditions in a tribological system defined by the Sommerfeld number, *S* [14,36,37,43,46]:

$$S = \frac{\eta v a}{L_n} \quad (1)$$

where η is the lubricant viscosity, *v* is the sliding speed, *a* is the contact width, and L_n is the normal load. When friction coefficients are plotted as a function of *S*, lubrication modes can be visualized. The curve takes the following form where μ_B and μ_{min} describe the maximum and minimum friction coefficients at boundary and elastoviscous modes, respectively, S_t describes the inflection point between modes, and *d* controls the rate of the transition:

$$\mu(S) = \mu_{min} + (\mu_B - \mu_{min}) e^{-(S/S_t)^d} \quad (2)$$

In the present study, a master Stribeck curve was calculated by setting μ_B=0.15, μ_{min}=0.05, S_t=2.74E-6, and *d*=0.62, which are similar to values described in a previous study [37]. The error between the measured synovial fluid friction coefficients and the master Stribeck curve was minimized while allowing η to vary. The η value at which the RMS error reached a minimum value was defined as the effective viscosity. The effect of exogenous HA, Hymovis®, on effective viscosity was calculated as the difference between effective viscosity with and without HA, and it is represented as $\eta_{eff,\pm HA}$.

4.3.4 Statistics

For all statistical tests, a p-value less than 0.05 was deemed significant. A linear mixed model was applied to determine changes in all measurements as a function of time with a random effect of patient to account for repeated measures. The fixed effect of time was either categorical (pre-operative, surgery, post-operative time-points) or continuous with time after ACLT. Analyses were performed in RStudio using the *lmer* and *emmeans* test packages followed by Tukey post-hoc tests [47,48]. Similarly, to determine the effect of exogenous HA on effective viscosity ($\eta_{eff,\pm HA}$

) and friction coefficient as a function of native lubricin or HA concentrations, a mixed model was specified with a fixed effect of HA or lubricin and a random effect of patient. The models' variance is reported by the marginal and conditional R^2, R_m^2 and R_c^2, respectively, which were determined in R using the *MuMIn* test package [49]. The variance explained by the fixed effects is R_m^2, while the variance explained by both the fixed and random effects is R_c^2. To satisfy the model assumptions of normality in the residuals, log transformations were applied to HA concentrations, weight average molecular weight ($\bar{M}_w$), number average molecular weight ($\bar{M}_n$), and effective viscosity with and without HA ($\eta_{eff,\pm HA}$). It was necessary to exclude data to achieve normality in the following statistical tests: HA concentration versus time and timepoint.

4.4 Results

4.4.1 Lubricin

Lubricin concentrations did not vary significantly with timepoint (Figure 4-1A, p=0.09) and were similar among pre-operative (3.15±1.38 mg/ml, Mean±SD), day of surgery (1.97±0.89 mg/ml, Mean±SD), and post-operative samples (1.69±1.01mg/ml, Mean±SD). Lubricin concentrations tended to decrease with time after ACLT excluding samples collected during post-operative visits (Figure 4-1B, $R_m^2 = R_c^2 = 0.31$, p=0.042).

4.4.2 HA

The concentration of HA varied significantly with timepoint (Figure 4-1C, p<0.0001). HA concentrations on the day of surgery (1.07±0.27 mg/ml, Mean±SD) were significantly higher than concentrations at the pre-operative (0.43±0.13 mg/ml, Mean±SD, p=0.0005 relative to surgery) and post-operative time points (0.20±0.06 mg/ml, Mean±SD, p<0.0001 relative to surgery). Pre-operative HA concentrations were greater than post-operative concentrations (p=0.019). Up to at least 60 days post-ACLT, HA concentrations were found to increase significantly with time after

injury (Figure 4-1D, R_m^2= 0.76, R_c^2=0.96, p=0.024). In two samples, HA concentrations were relatively high in this study and relative to other measurements of HA in post-traumatic synovial fluid [14,24]. It was necessary to exclude these measurements from the mixed model to achieve normally distributed residuals, and these exclusions are denoted in Figure 4-1C,D.

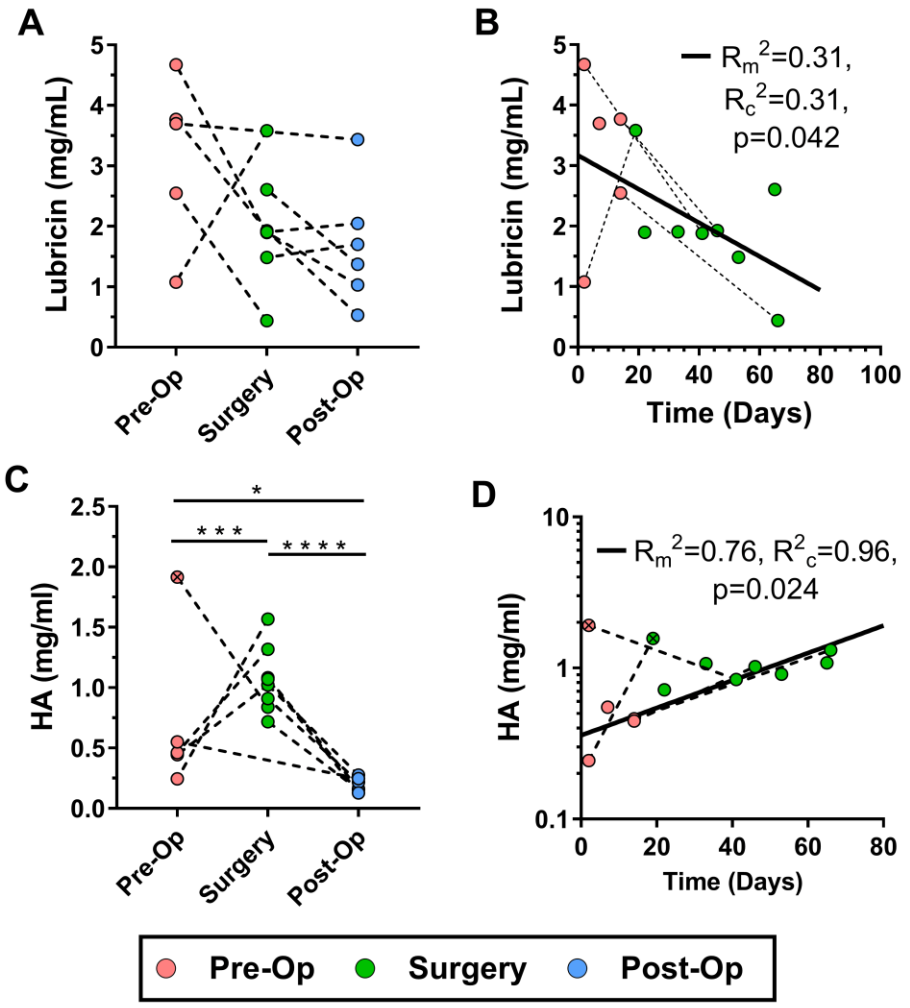

Figure 4-1: (A) Lubricin concentrations did not vary across timepoints (p>0.05, $n_{Pre\text{-}Op}$=5, $n_{Surgery}$=8, $n_{Post\text{-}Op}$=6) (B) but tended to decrease with time after ACLT from pre-operative to surgical timepoints (R_m^2=0.31, p=0.042). (C) HA concentrations were highest at the time of surgery compared to pre-operative (p=0.0005) and post-operative (p<0.0001 relative to surgery)

HA concentrations ($n_{Pre\text{-}Op}$=4-5, $n_{Surgery}$=8, $n_{Post\text{-}Op}$=6). (D) HA concentrations increased with time after ACLT from pre-operative to surgical timepoints (R_m^2=0.76, p=0.024). For (D), symbols with an "X" indicate data points that were excluded from the model to achieve normally distributed residuals.

The molecular weight distribution of HA varied with timepoint (Figure 4-2). The distribution of HA was most polydisperse in post-operative samples (Figure 4-2F, PDI=2.24±0.07, Mean±SD) as compared to pre-operative (PDI=1.98±0.12, Mean±SD, p=0.0002) or day of surgery (PDI=1.89±0.1, Mean±SD, p<0.0001) samples. In contrast, the weight average molecular weight ($\bar{M}_w$) and number average molecular weight ($\bar{M}_n$) did not vary with timepoint (Figure 4-2D, E, p=0.69 and p=0.17, respectively). Weight average molecular weights were 3.08±0.68MDa, 2.88±0.72MDa, and 2.83±0.38MDa for pre-operative, day of surgery, and post-operative samples, respectively (Mean±SD). Number average molecular weights were 1.56±0.36MDa, 1.54±0.44MDa, and 1.27±0.18MDa for pre-operative, day of surgery, and post-operative samples, respectively (Mean±SD).

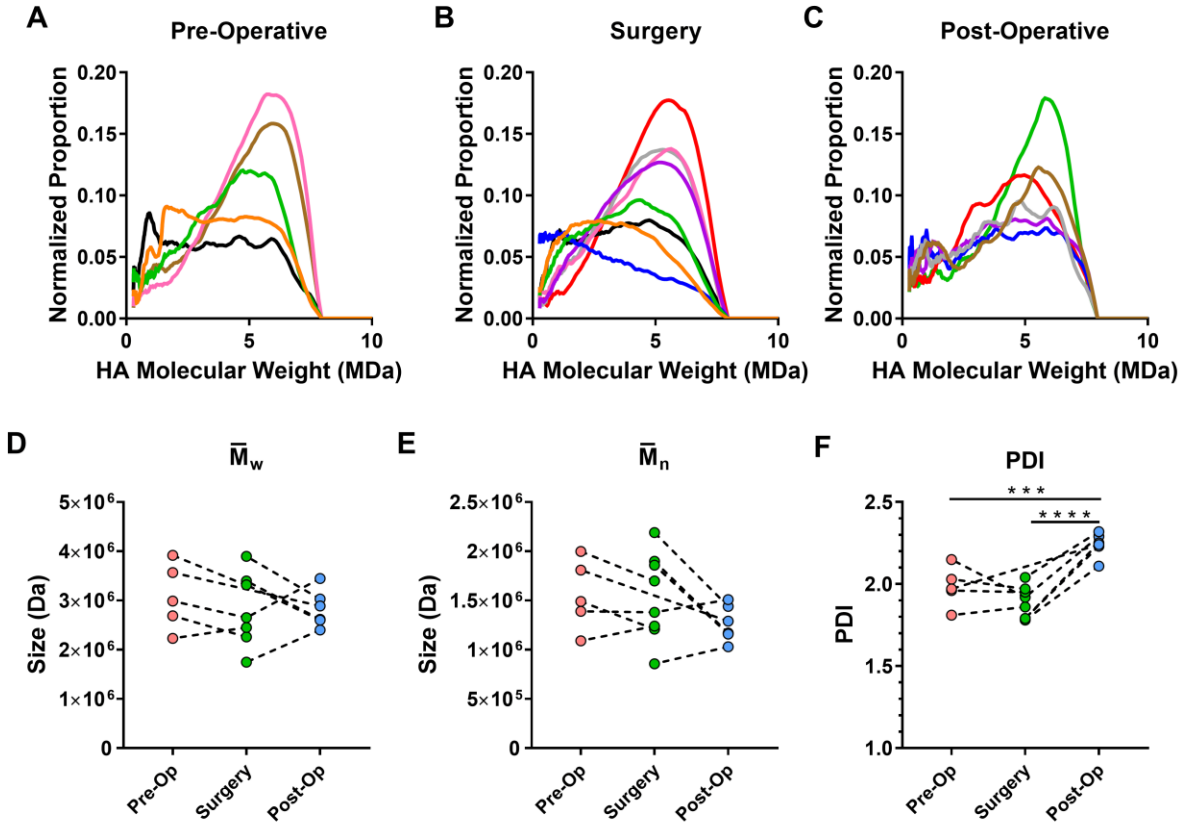

Figure 4-2 : The normalized proportion vs. HA molecular weight is shown for pre-operative (A), surgical (B), and post-operative (C) timepoints ($n_{Pre\text{-}Op}$=5, $n_{Surgery}$=8, $n_{Post\text{-}Op}$=6). Each color in (A-C) represents a unique patient. The weight average molecular weight (D, $\bar{M}_w$) and number average molecular weight (E, $\bar{M}_n$) did not vary with timepoint (p=0.69 and p=0.17, respectively), but the polydispersity index (PDI) was significantly higher post-operatively than at pre-operative (p=0.0002) or surgical timepoints (p=<0.0001).

4.4.3 Friction

Friction coefficients of synovial fluid exhibited the most changes at 0.1mm/s compared to higher speeds. However, friction coefficients did not vary significantly with timepoint (Figure 4-3A, p=0.89). Mean friction coefficients were similar for pre-operative (0.14±0.03, Mean±SD), day of surgery (0.14±0.05, Mean±SD), and post-operative (0.15±0.06, Mean±SD) samples. Furthermore, there was no relationship between friction coefficient and time after ACLT (Figure 4-3B, R_m^2= 0.001, R_c^2=0.44, p=0.91).

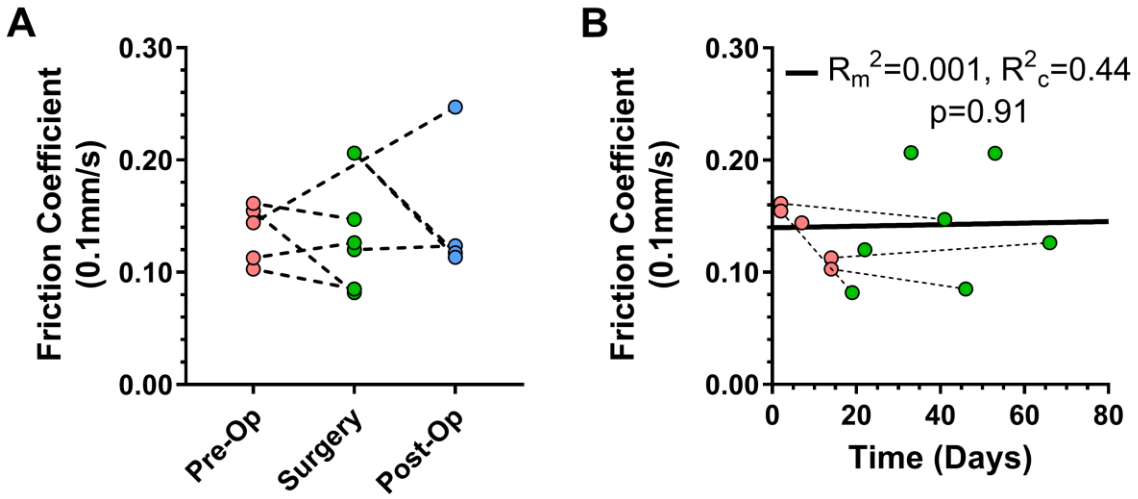

Figure 4-3 : (A) Friction coefficients did not vary with timepoint (p=0.90) nor with time after ACLT (B) ($n_{Pre\text{-}Op}$=5, $n_{Surgery}$=7, $n_{Post\text{-}Op}$=4).

Effective viscosities tended to increase with the addition of HA, but ranged from a decrease of -2.52 Pa·s to an increase of 17.8 Pa·s. Effective viscosities of pure synovial fluid did not vary with timepoint ($p=0.96$). The effect of HA on effective viscosity did not vary with timepoint (Figure 4-4A, $R_m^2= 0.26$, $R_c^2=0.77$, $p=0.24$). Stribeck curves showed that synovial fluids with HA (Figure 4-4C) were shifted towards higher Sommerfeld numbers and were more concentrated in the mixed and elastoviscous modes than synovial fluids without HA (Figure 4-4B). The change in effective viscosity appeared to depend not on lubricin (Figure 4-4D, $R_m^2= 0.08$, $R_c^2=0.21$, $p=0.35$), but rather on the native concentration of HA (Figure 4-4E, $R_m^2= 0.38$, $R_c^2=0.74$, $p=0.013$).

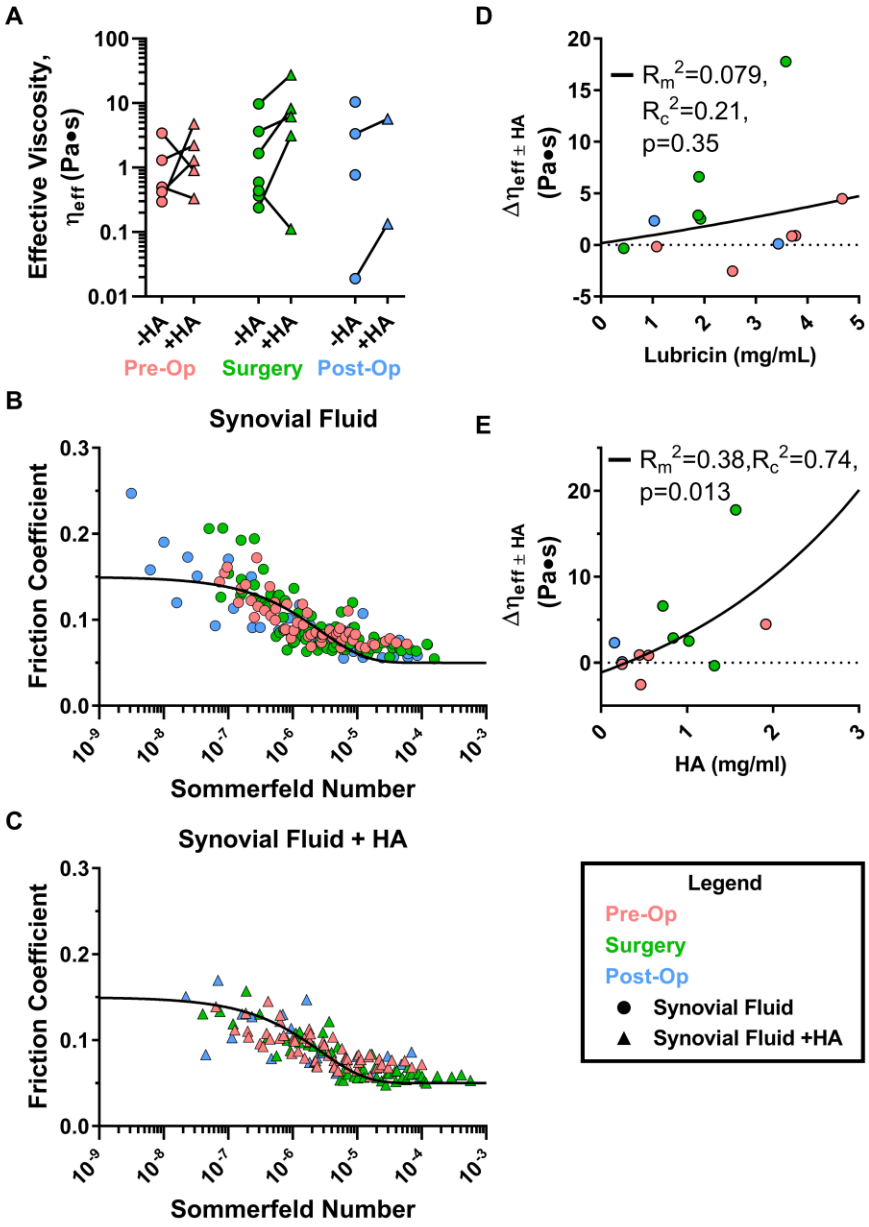

Figure 4-4: (A) Effective viscosities of pure synovial fluid did not vary with timepoint and nor did the change in effective viscosity after viscosupplementation (-HA: $n_{Pre\text{-}Op}$=5, $n_{Surgery}$=7, $n_{Post\text{-}Op}$=4, +HA: $n_{Pre\text{-}Op+HA}$=5, $n_{Surgery+HA}$=5, $n_{Post\text{-}Op+HA}$=2). (B) Synovial fluid samples fit well to a master Stribeck curve and operated across all lubrication modes. (C) When mixed with the viscosupplement, samples tended to operate more in the mixed and elastoviscous lubrication modes. (D) The change in effective viscosity with the viscosupplement did not depend on lubricin

(n_{total}=12), but rather on native HA concentrations (p=0.013, n_{total}=12).

The addition of HA to the synovial fluids resulted in either no change or a decrease in friction coefficients at 0.1mm/s sliding speed (Figure 4-5A). On average, HA reduced friction coefficients by -0.029±0.028, -0.022±0.028, and -0.053±0.061 for pre-operative, day of surgery, and post-operative samples, respectively (Mean±SD). Overall, the effect of HA on friction coefficient did not vary with timepoint (p=0.46). Friction coefficients of synovial fluid mixed with HA were normalized to the friction coefficient of synovial fluid alone. The percent reduction of friction by exogenous HA was weakly correlated with lubricin concentration (Figure 4-5B, R_m^2= 0.23, R_c^2=0.81, p=0.026), but showed no dependence on HA concentration (Figure 4-5C, R_m^2= 0.04, R_c^2=0.65, p=0.40). Friction coefficients of pure synovial fluid showed no dependence on lubricin or HA concentration (p>0.05, data not shown).

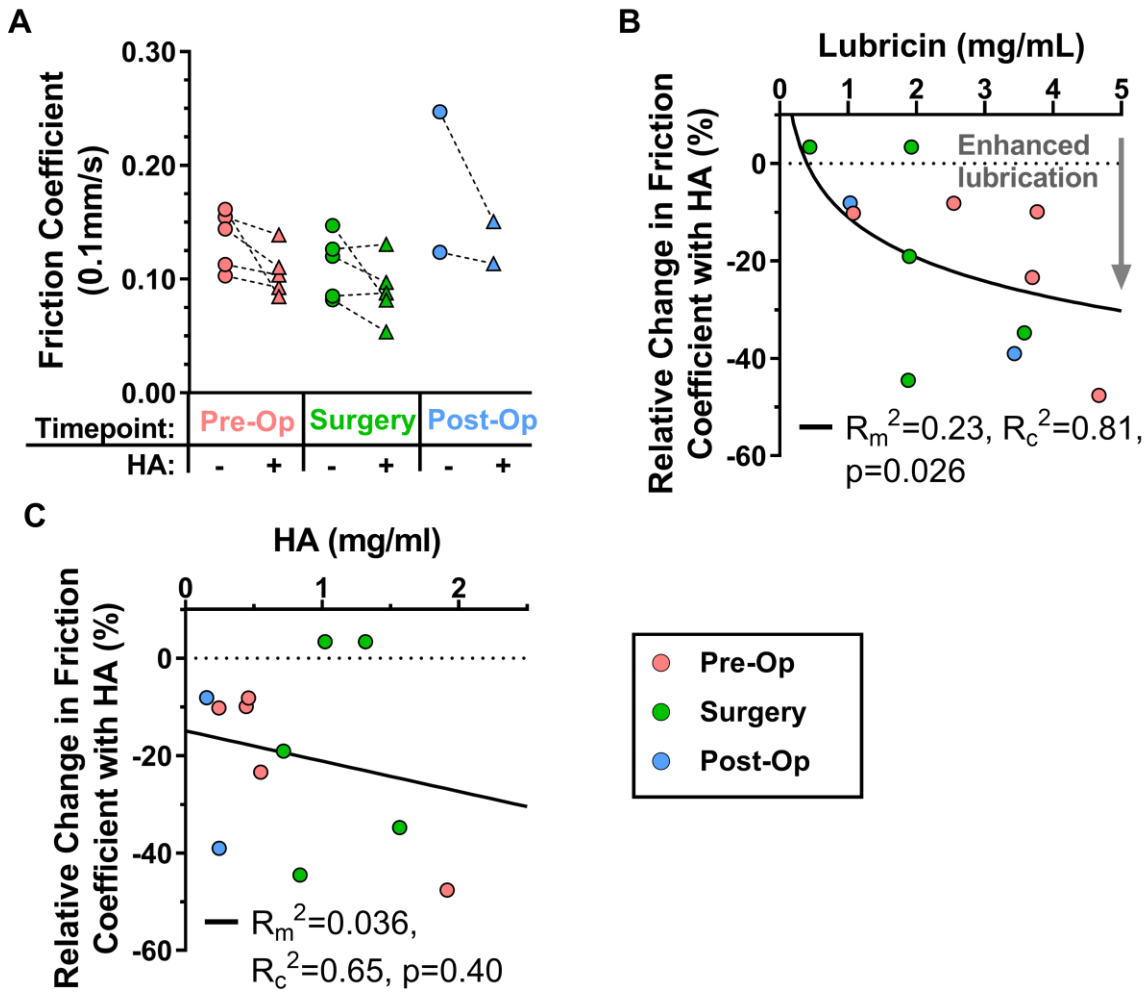

Figure 4-5: (A) SF mixed with exogenous HA (Δ) resulted in lower friction coefficients (0.1mm/s sliding speed) compared to SF alone (○). The lubricating potency of HA was similar across timepoints (p=0.46, $n_{Pre\text{-}Op}$=5, $n_{Surgery}$=5, $n_{Post\text{-}Op}$=2). (B) Lubrication by exogenous HA was modulated by the concentration of lubricin in SF (R_m^2=0.23, p=0.026, n_{total}=12), but (C) did not depend on the concentration of HA (R_m^2=0.04, p=0.40, n_{total}=12).

4.4.4 Cytokines

Of the measured cytokines and enzymes, only IL-1RA, MMP-3, IL-6, and MCP1 varied with time (Figure 4-6, Table 4-1). Pre-operative and surgical samples exhibited similar mean concentrations of IL-6, MCP-1, IL-1RA, and MMP-3 ($p>0.05$). For IL-6, IL-1RA and MMP-3, they were both significantly lower than post-operative concentrations (pre-operative vs. post-operative: $p=0.027$, $p=0.0016$ and $p=0.0036$, respectively and surgery vs. post-operative: $p=0.0007$, $p=0.0006$ and $p=0.0044$, respectively). For MCP-1, only concentrations at the time of surgery were significantly lower than pre-operative concentrations ($p=0.035$).

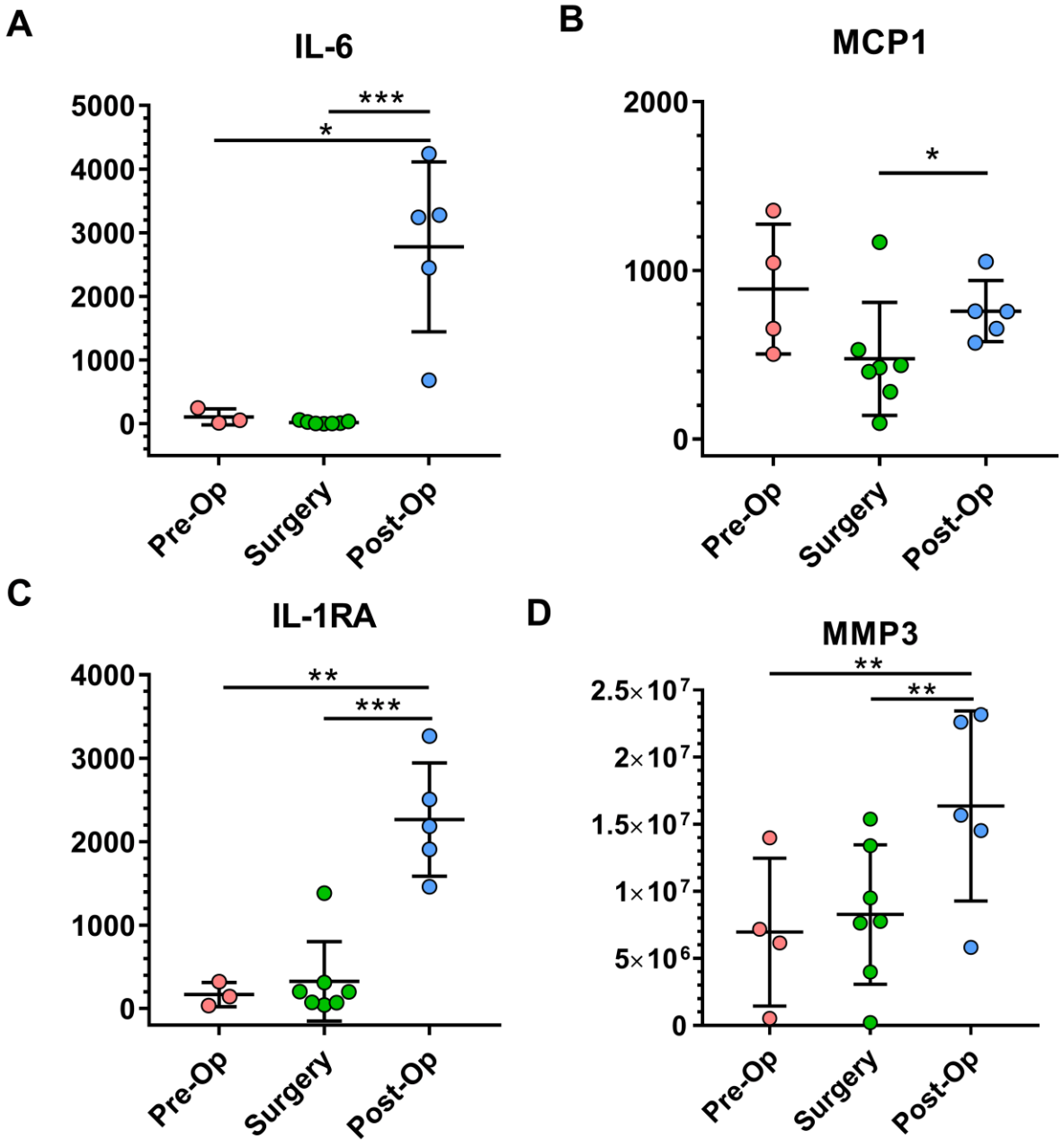

Figure 4-6: Concentrations of IL-6 (A), MCP-1 (B), IL-1RA (C), and MMP-3 (D) in pg/ml at the post-operative timepoint were consistently higher than pre-operative and surgical timepoints. The pre-operative and surgical timepoints exhibited similar concentrations of these cytokines and enzymes ($n_{Pre\text{-}Op}$=3-4, $n_{Surgery}$=7, $n_{Post\text{-}Op}$=5).

Table 4-1: Cytokine Concentrations by Group

Timepoint:	A. Pre-Op		B. Surgery		C. Post-Op		p-values		
Marker	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**A vs B**	**A vs C**	**B vs C**
IL-6 (pg/ml)	108.00	125.70	21.54	21.69	2778.46	1333.09	0.29	*0.027*	*0.0007*
MCP-1 (pg/ml)	890.00	385.20	476.30	335.30	759.0	181.6	0.12	0.84	*0.035*
IL-1RA (pg/ml)	168.30	144.50	327.20	475.50	2266.0	678.6	0.96	*0.0016*	*0.0006*
MMP3 (µg/ml)	6.96	5.52	8.27	5.20	16.36	7.08	0.52	*0.0036*	*0.0044*

4.5 Discussion

In this study, we sought to determine temporal variations in synovial fluid lubrication following ACLT and ACL reconstruction by characterizing its composition and frictional properties. With respect to composition, HA concentrations and HA molecular weight exhibited the most significant temporal variations. HA concentrations increased between the pre-operative and surgical timepoints but plummeted by the post-operative visit to pre-operative concentrations. HA polydispersity increased at the post-operative timepoint relative to pre-operative and surgical polydispersities, which were similar. Surprisingly, lubricin concentrations were similar among timepoints and tended to decrease between the pre-operative and surgical timepoints. Despite the changes in HA and lubricin in the synovial fluid, friction coefficients did not vary significantly with time. Exogenous HA generally increased effective viscosity and resulted in a -0.03 mean reduction in friction coefficient relative to pure synovial fluid. These responses in effective viscosity and friction coefficient were dependent on native HA and lubricin concentrations, respectively. While viscosupplements were developed originally to restore HA concentrations and improve lubrication, their lubricating efficacy may not depend solely on HA but also lubricin.

The temporal pattern of HA variation following ACLT agrees with previous studies of ACLT in other species and measurements in other joint traumas like articular fracture. Healthy synovial fluid concentrations of HA are generally accepted to occur in the range of 1-4 mg/ml [16,50–54]. Joint disease and trauma results in a decrease in HA concentrations to <1mg/ml and a shift toward lower molecular weight polymer [14,16,20,22,53,55,56]. In the present study, HA concentrations pre-operatively (0.43±0.13 mg/ml) and post-operatively (0.20±0.06 mg/ml) were similar to concentrations measured in the aforementioned studies. The low post-operative concentrations were likely due to synovial fluid lavage during arthroscopic surgery, which has

been of focus of recent investigations linking lavage to a loss of synovial fluid lubricants and increased cartilage friction [14,57,58]. Concentrations of HA between the pre-operative and surgical timepoints recovered toward more normal mean concentrations of 1.07±0.27 mg/ml. There are several explanations for this response including: (1) reduced HA degradation, (2) increased HA synthesis, (3) reduced effusion volumes, or (4) reduced HA clearance from the joint. Per (1), HA degradation would be promoted by a pro-inflammatory joint environment. The synthesis of HA by HA synthases on synoviocytes is promoted by TGF-β and IL-1β, which were not quantified in this study [59]. Of the many cytokines and enzymes profiled in this study, no differences were detected between the pre-operative and surgical timepoints. Therefore, we believe it is unlikely that reduced HA degradation or increased HA synthesis is responsible for the recovery of HA at the time of surgery. There is more evidence to suggest that HA clearance rates decrease between pre-operative and surgical timepoints. For example, in a rabbit ACLT model, low molecular weight HA exhibited lower clearance rates at 28 days post-ACLT compared to 7 days post-ACLT [60].

Lubricin concentrations did not vary significantly with time, which contributes to a growing body of evidence that the patterns of lubricin concentrations following traumatic joint injury are highly variable. It is worth noting that supraphysiological concentrations of lubricin were reported in this study, which could be due to the relatively high dilution factor of the ELISA (8,000x) as well using synovial fluid-purified, bovine lubricin as a standard. Therefore, our reported absolute concentrations should only be interpreted in the context of this study. Examining the pattern of lubricin concentrations with time, there was a trend towards decreasing concentrations of lubricin with time after ACLT, which contrasts with a previous study on human ACLT, which observed an increase in lubricin over the course of a year after ACLT [31]. One

major difference between our study and Elsaid et al. is the duration over which lubricin was measured; in the present study, synovial fluid concentrations were only measured up to approximately 65 days after ACLT. When the lubricin vs. time post-ACLT data from Elsaid et al. is truncated to this timeframe, it is unclear how lubricin varies. Indeed, the temporal variation in lubricin concentrations may not be linear as our group has previously observed in equine articular fractures cases [14,21]. An ovine ACLT model observed an increase in lubricin early after ACLT compared to 20 weeks post-ACLT [24] and contralateral control limbs [61]. In three of four patients with measurements at both the pre-operative and day of surgery timepoints, there was a decrease in lubricin concentrations. Human patients are extremely variable, and it is possible factors like body mass index, age, race, and exercise could be modulating lubricin concentrations. Animal studies enable better control over inter-subject variation, but do not necessarily capture realistic human variation. Moreover, naturally-occurring and surgically-induced ACLT may differ significantly in severity and trauma to the joint.

Perhaps the most surprising result from this study was that the effect of viscosupplementation on friction coefficients at low sliding speeds and effective viscosity depended on native synovial fluid composition. Interestingly, increased concentrations of lubricin were correlated with enhanced lubrication by exogenous HA. This correlation was relatively weak; according to the mixed model, only 23% of the variation in lubrication by HA depended on lubricin and nearly 60% was due to patient variability. Similarly, there was a moderate and positive correlation between the change in effective viscosities after exogenous HA treatment with native HA. The reduced friction coefficients at 0.1mm/s and increase in effective viscosity suggest that the viscosupplement transitions the system towards higher Sommerfeld numbers and further into mixed and elastoviscous lubrication modes. While friction coefficient and effective viscosity

changes depended on different lubricants, i.e., lubricin or HA, respectively, it is likely that both lubricin and HA contribute to the effectiveness of the viscosupplement but our model was unable to detect this due to low sample size and high patient variability. The synergistic relationship between lubricin and HA on cartilage lubrication is well-recognized [36,62,63]. The premise of viscosupplementation therapy is based on the loss of HA, viscosity, and viscoelasticity in OA. The results from this study bolster the theory that lubricin is necessary to localize HA to the cartilage surface. Therefore, viscosupplementation should be considered alongside both lubricin and HA content in the joint.

This study is not without limitations. The most significant limitation is the lack of heathy control SF for comparison of our results. Inherently, human studies of PTOA do not allow for the collection of SF at baseline, and so surrogates such as SF from healthy, contralateral joints or healthy animal sources must be considered. Without such control fluid, it is not possible to say whether our reported results represent a shift above or below that of a healthy joint. Also, our sample size was relatively small, which limited the power of our model to detect how changes in lubrication varied with the composition of synovial fluid. Another limitation is that we did not examine the molecular weight or glycosylation of lubricin, which are both vulnerable in the post-traumatic joint environment where lubricin may be of a lower molecular weight or exhibit increased sialylation [64,65]. Altered glycosylation is known to affect lubricin's cartilage lubricating ability [66,67].

In conclusion, we have determined the variation in synovial fluid composition and lubricating properties following ACLT and ACLR. To our knowledge, this is the first time this analysis has been performed for human synovial fluid samples. The most dramatic changes in composition occurred in the concentrations of HA. Similar to other studies of the post-traumatic

synovial environment, HA concentration were low immediately after injury and recovered with time towards more normal levels. However, HA polydispersity was similar immediately following ACLT and at the time of surgery weeks later but increased following ACLR. Lubricin concentrations did not vary significantly with time but tended to decrease with time after ACLT. Despite the compositional variations, friction coefficients and the effective viscosity of synovial fluids did not vary with timepoint. However, the lubricating ability of in vitro HA viscosupplementation depended on native synovial fluid composition. Lubricin concentrations modulated the effectiveness of HA viscosupplementation, whereby increased concentrations of lubricin resulted in larger decreases in friction coefficient. While viscosupplementation therapy could be beneficial for improving joint lubrication after ACLT, its effectiveness in reducing friction is dependent on synovial lubricin and HA concentrations. This dependency may explain clinical variability in the efficacy of viscosupplementation and may be useful for clinicians when considering viscosupplementation therapy.

4.6 Acknowledgements

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CHAPTER 5
Conclusions and Future Directions

5.1 Conclusions

While synovial lubrication has been studied for over 100 years, the link between synovial fluid composition and mechanical properties is unclear. Previous research has primarily focused on the key lubricants, lubricin and hyaluronic acid, in isolation. Importantly, they show varying changes in disease and function synergistically to lubricate cartilage. Furthermore, it is accepted that the joint operates in three lubrication regimes dominated by unique lubrication mechanisms. This behavior manifests as a nonlinear relationship in the magnitude of friction coefficient as a function of the joint's operating conditions, which can be described by the Stribeck curve. However, the cartilage lubrication field has not widely adopted the application of the Stribeck framework. In studies comparing synovial fluid or model fluids (i.e., lubricin solutions, HA solutions, etc.), friction coefficient is presented for a single speed or set of speeds without accounting for important factors like the contact load or differing lubricant viscosities. Interpretation and comparison of the results between studies is challenged by the fact that research groups use various types of tribometers with different materials, motion profiles, and contact geometries. Thus altogether, our understanding of multi-modal friction properties is limited.

The goal of this dissertation research was to determine holistically how osteoarthritis affects synovial fluid's lubricating properties. Broadly, lubrication and composition varied with both arthritis endotypes and time following traumatic joint injury. In Chapter 2, we studied how intra-articular fracture affected synovial lubrication in equines. We conducted serial measurements from baseline pre-injury values to 10 weeks post-operatively of lubricin and HA concentration, HA molecular weight, viscosity, and multi-modal friction coefficients of synovial fluid in both the

injured and unaffected limbs. In Chapter 3, we examined the differences in lubrication and composition between two patient cohorts with non-inflammatory osteoarthritis or inflammatory arthritis. Additionally, we examined how viscosupplementation with HA differentially affected lubrication. In Chapter 4, we determined how synovial fluid composition and lubrication was altered following anterior cruciate ligament tear (ACLT) injuries and ACL reconstruction in human patients. The effect of viscosupplementation on synovial fluid collected at these different timepoints and as a function of synovial fluid composition was determined.

In Chapter 2, we found distinct, time-dependent changes in composition and lubrication following articular fracture. Lubricin concentrations increased while HA concentrations plummeted. Relative to pre-injury values, these responses were significantly different for four weeks after fracture. Synovial fluids also exhibited low viscosity for five weeks after injury. Interestingly, while we hypothesized that the increase in lubricin would produce lower boundary mode friction coefficients, no changes were seen. This could be due to the concentration of lubricin exceeding saturating levels for lubrication. Stribeck analysis using the measured viscosities indicated that instead of boundary or elastoviscous mode friction coefficients, the transition number reflecting the inflection point between these modes was reduced after fracture. A lower transition number reflects enhanced lubrication as the tribological system transitions earlier from high-friction boundary mode to low-friction boundary mode.

In Chapter 3, we found that synovial fluid from inflammatory arthritis and non-inflammatory OA patients exhibited distinct tribological endotypes. Using Stribeck analysis, a subset of inflammatory arthritis synovial fluids exhibited aberrant behavior distinct from other inflammatory arthritis fluids and non-inflammatory OA fluids with typical tribological behavior. This was not explained by viscosity, which were similar across samples as measured by rotational

rheometry. Aberrant behavior was characterized by high and variable friction coefficients, which increased with sliding speed, a behavior that has not been previously observed. Additionally, aberrant samples had low lubricin concentrations and effective viscosities. When mapped onto a master Stribeck curve, aberrant fluids tended to operate in high-friction boundary mode unlike fluids with typical tribological behavior that operated in the mixed and elastoviscous modes. Importantly, HA viscosupplementation did not eliminate aberrant tribology. We hypothesize that the ineffectiveness of the viscosupplement in reducing friction and aberrant behavior is due to a low amount of lubricin and a consequent hindering of viscous lubrication mechanisms.

In Chapter 4, we found that synovial HA concentrations varied significantly following ACLT and ACLR in human patients, while lubricin concentrations exhibited no differences across timepoints. Despite the change in HA concentration with time, friction coefficients did not vary with time. Similarly, friction coefficients did not show a significant correlation with the amount of lubricin or HA in synovial fluid. Importantly, in vitro HA viscosupplementation affected lubrication in a composition-dependent manner. The change in effective viscosity and friction coefficient with viscosupplementation depended on native HA and lubricin concentrations, respectively. Increased concentrations of either led to enhanced lubrication by the viscosupplement.

As a whole, the major contributions of this dissertation research to the field of cartilage lubrication are as follows:

Application of the Stribeck framework to Synovial Lubrication: In the three studies included in this work, the Stribeck framework was applied for the first time to the study of synovial fluid's lubricating properties. In Chapter 2, we saw that articular fracture altered the transition number describing the point at which a system switches between high-friction, boundary mode

and low-friction, elastoviscous mode. In Chapter 3, an aberrant tribological behavior was observed where friction increased with Sommerfeld number, which is the first time such a response has been described to our knowledge. In both Chapters 3 and 4, application of the Stribeck framework to calculate the effective lubricating viscosity enabled visualization of individual lubricants' position on the Stribeck curve. Consequently, the dominant operating regimes for the lubricants could be compared. For example, in Chapter 3, inflammatory arthritis synovial fluids operated predominantly in the boundary mode, while fluids with more typical tribological behavior operated in the mixed and elastoviscous modes. Together, these studies demonstrate that the Stribeck framework can be successfully used to describe synovial fluid lubrication. It proved a valuable tool for discerning changes to lubrication in disease as a function of time in post-traumatic osteoarthritis or arthritis phenotype. Finally, it is a unifying framework for studying lubrication that enables comparison among studies, unlike measurements of friction coefficient, which vary with tribometer type and are typically reported as a function of speed. Furthermore, it permits the detection of lubrication mode-specific changes. In the context of disease and therapy, this could be important for improving lubrication-based therapies to target mode-specific changes.

Holistic Analysis of Lubricant Composition and Mechanics: In each of the studies, synovial fluid lubrication was analyzed in terms of viscosity, multi-modal friction properties, and lubricin and HA content. These studies reflected several key knowledge gaps in cartilage lubrication.

First, the pathogenesis of post-traumatic osteoarthritis and the role of lubrication in this process is poorly understood. Chapter 2 and 4 expand our knowledge of the temporal changes in synovial fluid composition and mechanics. In Chapter 3, we also showed that there are unique endotypes of arthritis characterized by aberrant tribology and we linked this to a low amount of

lubricin. Together these studies present an expansive view of the spectrum of synovial fluid changes in disease.

Second, most studies of osteoarthritis examine either HA or lubricin, and rarely both lubricants. Though both lubricants have long been recognized for their critical role in cartilage lubrication, the synergistic interaction between them in reducing friction is a more recent concept. In this dissertation, all studies examined both lubricin and HA, and their relationship to lubrication.

Quantification of Effective Viscosity: Effective viscosity proved a useful tool in Chapters 3 and 4 for comparing lubricants. It has only been applied to cartilage lubrication in one recent study of viscosupplements [1]. In Chapter 3, both traditional rheology and effective viscosity calculation were performed. Surprisingly, no difference in viscosity as measured by rotational rheometry was discovered between our inflammatory and non-inflammatory arthritis cohorts or fluids with aberrant versus typical tribology. However, effective viscosity showed that aberrant fluids exhibited lower effective viscosities than fluids with typical tribological behavior. Effective viscosities may better capture viscous behavior in cartilage lubrication than traditional rheology. Current rheology techniques such as those used in Chapter 2 fail to capture surface interactions between synovial fluid and the cartilage surface, are subject to systematic error resulting from interfacial effects, and require a fixed gap between surfaces. Additionally, we found that effective viscosities were not significantly altered by the addition of a viscosupplement, whereas viscosity increased by a few orders of magnitude. Low effective viscosities equate to lubrication in the high-friction boundary mode. Thus, in the context of studying cartilage lubrication, improving viscosity alone in insufficient. It is necessary to enhance the effective viscosity to achieve adequate lubrication. This result adds to the previous study from the Bonassar group demonstrating that

improvements in clinical outcome, WOMAC score, were positively correlated with effective viscosity but showed no relation to viscosity or viscoelasticity measured via traditional methods.

Moving forward, cartilage lubrication should be described by effective viscosity. In Chapters 2 and 3, use of the bulk viscosity through measurements on a rotational rheometer lead to questionable Stribeck curves. For example, in Chapter 2, decreased zero-shear viscosity in disease translates to an earlier transition to low-friction elastoviscous mode, which is a counter-intuitive result. A similar problem was reported by Cassin et al. in describing lubrication of water and guar gum solutions; use of the zero-shear rate viscosities erroneously suggested that water formed hydrodynamic films before guar gum, but this problem was overcome by use of effective viscosities [2]. While in Newtonian fluids, Stribeck curves can readily be created to describe friction as a function of either film thickness or the product of entrainment speed and viscosity, this is not the case for non-Newtonian fluids like synovial fluids and HA. In such cases, the effective shear rate and film thickness are unknown. The compliant and porous nature of cartilage further complicates the estimates of effective shear rate and film thickness. In this thesis work, effective viscosity was calculated based on the friction data being mapped onto a master Stribeck curve spanning all lubrication modes. One surprising outcome in Chapter 3, especially, was that HA viscosupplements did not significantly alter the effective viscosities. Given this, is it appropriate to measure synovial fluid or viscosupplement viscosities with a rheometer? In the context of cartilage lubrication by these fluids, the answer is arguably no because large increases in viscosity do not always translate to improved friction.

It is also worth considering the value of rheology and how it is used for cartilage lubrication. A recent study of soft, PDMS contacts lubricated by a shear-thinning solution estimated shear rates in the contact zone were on the order of $1x10^4$ s^{-1} and pointed out they were

beyond the sensitivity of a common, commercial rheometer [3]. While the shear-thinning curves obtained from rheometry possessed some value in modeling lubrication in cartilage contacts, this information suggests new methods to measure viscosity accurately at these shear rates should be developed. This adds to the other limitations of traditional rotational rheometers that use inert metal surfaces, have fixed gaps, and are prone to interfacial artifacts. Altogether, traditional rheology techniques may not accurately capture fluid behavior in a cartilage-based, tribological system.

The mathematical techniques and assumptions made to calculate effective viscosities should also be evaluated to optimize its accuracy and applicability. Broadly in the field of tribology, the calculation of effective viscosity for shear-thinning materials in soft contacts is an active area of research. In review of present work, is it appropriate that the calculation of effective viscosity span the entire Stribeck curve? Other studies of effective viscosity have either assumed a collapse of the data at the hydrodynamic or mixed regimes when viscous effects govern formation of a fluid film and friction coefficient [2,4] or mapped the data onto a master Stribeck curve for a Newtonian fluid [5]. For cartilage, should we instead assume superposition in the elastoviscous regime to allow for variation in friction between lubricants at the boundary and mixed modes? We should continue to look to theories and methods proposed by tribologists and adapt them appropriately to describe cartilage lubrication as accurately as possible.

Effect of Viscosupplementation Depends on Synovial Fluid Composition: For some osteoarthritis patients, viscosupplements provide long-lasting reductions in pain and restored joint function. However, the heterogeneity in patient outcomes following viscosupplementation is well-recognized by the orthopedic community, but its causes remain unclear. While many factors have been considered such as the severity of OA or patient age, our research suggests that the native

synovial composition may modulate the mechanical response to synovial fluid lubrication. As mentioned previously, lubricin and HA act synergistically within the joint. One of the prevailing theories is that lubricin and HA form physical entanglements that increase viscosity at the cartilage surface and thereby facilitate a transition towards low-friction modes via viscous lubrication mechanism. While the concept of synergism is not novel, we are the first to suggest that this synergy may affect viscosupplementation and to provide in vitro data supporting this hypothesis using clinical samples as well as physiologically relevant, cartilage-based tribology measurements. The effect of composition on viscosupplementation was explored in Chapters 3 and 4. The consequence of this result is significant to viscosupplement therapy as HA may require sufficient lubricin to reduce friction. Thus, future viscosupplements should be developed with this synergism in mind, for example, by altering the formulation to include lubricin or enhancing lubricin-HA interactions.

5.2 Future Directions

There are several opportunities for continued research related to this thesis, which center on enhancing our understanding of cartilage lubrication and applying that knowledge to advance therapeutics for OA.

Mapping Lubricin-HA Synergy in the Stribeck Curve: Synovial fluids are complex biological tissues comprised of much more than lubricin and HA, which were the focus of this dissertation research. While the Stribeck framework proved a useful tool for analyzing lubrication, there is a strong need to establish an idealized Stribeck curve for synovial fluid. In studies of clinical samples, it would be helpful to ground changes in lubrication to such a model, especially given the wide variability among patients. Experiments should cover a range of physiologically relevant concentrations of lubricin and HA that reflect health and disease. Likewise, HA shifts

toward lower molecular weights in disease, and therefore this should also be varied when generating the model Stribeck curve. Presently, Stribeck curves have been characterized for only a single concentration of lubricin with HA solutions of varying viscosity [6]. In studies that have not used the Stribeck framework, measurements of boundary mode cartilage friction coefficients with lubricin and HA suggest that their relationship is complex and inter-dependent [7–9]. For example, while friction coefficients were measured to decrease with HA molecular weight, the addition of lubricin at moderately high concentrations of 450μg/ml eliminated the molecular-weight dependence [7]. With our present understanding of lubricin-HA synergy, it is likely that in the presence of HA, increasing amounts of lubricin would result in a lowering of boundary mode friction coefficients as well as an earlier and sharper transition to elastoviscous mode. Furthermore, increasing amounts of HA or higher molecular weight forms would increase viscosity, and by the theory of viscous lubrication, move the system along the path of the Stribeck curve. Because increased friction can predispose cartilage to degradation in OA [10,11], it is important to understand how lubricant composition affects cartilage friction coefficients and the transitions from high-friction boundary mode to low-friction elastoviscous mode.

Probing the Mechanisms of Lubricin-HA Interactions: There are several proposed mechanisms for lubricin-HA's physical interactions including (1) electrostatic attraction, (2) hydrophobic interactions, (3) hydrogen bonding, and (4) steric entanglement. The effect of each of these mechanisms on lubricin-HA synergy and lubrication is unknown. Future studies could explore the impact of these mechanisms by altering the composition of the lubricant bath in the following ways:

(1) Electrostatic Attraction: The somatomedin-B-like and hemopexin-like domains of lubricin contain some positive charges [12] and thus have the potential to interact with HA, which

exhibits a negative charge from its carboxylic acid groups. Altering the salt concentration or pH of the bath would modulate the degree of charge shielding around lubricin and HA. By increasing the salinity, electrostatic attraction would be reduced between lubricin and HA. Control measurements of lubricin and HA alone would be critical as altering the salinity can change the conformation of the molecules and possibly interactions with the cartilage surface [13].

(2) Hydrophobic Interactions: Non-polar regions of HA may also interact with the globular, hydrophobic somatomedin-B-like and hemopexin-like domains at the end of lubricin [12]. These interactions could be disrupted by the addition of a non-ionic surfactant such as Triton^{TM}-X-100.

(3) Hydrogen bonding : Hydrogen bonding may also occur between lubricin and HA [14]. Such interactions could be altered through the addition of chaotropic agents to the bath to disrupt the hydrogen bonding. Notably, even low concentrations of chaotropic agents can disrupt protein structures and alter hydrophobic interactions.

(4) Steric entanglement: Molecular entanglement between lubricin and HA may support the formation of lubricin-HA gel layer at the cartilage surface [6,15]. Increasing the temperature of the bath would increase the thermal motion of the polymers and thus reduce the degree of entanglement.

An enhanced understanding of how inter-molecular interactions contribute to friction would enable to design of new materials. Additionally, basic biophysical properties of the synovial fluid in disease versus health have not been heavily reported on, but slight changes could have significant effects on molecular interactions and conformation [13].

Enhancing Interactions Between Boundary Lubricants and HA: Lubricin-HA synergy in lubrication arises from their physical entanglement at the cartilage surface [6,7,16–19]. Given

the wide spectrum of lubricin and HA concentrations that occur in disease, exploiting and enhancing this synergy could lead to enhanced lubrication and joint health. Recently, a polymer-peptide coating strategy was designed to bind HA and localize it to cartilage and ocular surfaces [20]. The polymer peptide consisted of an HA-binding peptide (amino acid sequence: GAHWQFNALTVR), polyethylene glycol linker, and a collagen binding peptide for surface targeting. It enhanced HA retention in the joint by over ten-fold, and following incubation with HA and washout with PBS, friction coefficients were maintained at the same magnitude as HA in solution. Harnessing recent developments in producing recombinant lubricin [21], one or several HA binding peptides could be inserted into lubricin. One advantage of the HA-binding peptide is its length: 12 amino acids. Thus, its insertion would not dramatically affect the 1,404 amino acid long lubricin molecule. Lubricin's C-terminus and mucinous domain are essential to its cartilage-binding and boundary-lubricating properties, but the N-terminus' role is less clear with current theories suggesting it can dimerize with other lubricin molecules [12,22,23] In terms of initial molecular design, placement between the N-terminus and mucinous domain is an optimal region for insertion. This concept could be extended to lubricin-mimetics as well [24–26]. Ultimately, leveraging lubricin-HA synergy to design new lubricants could represent a new therapeutic for treating osteoarthritis.

Visualizing Fluid-Film Lubrication: Early research into cartilage lubrication advocated the theory that cartilage is lubricated by the formation of fluid films [27,28]. In more recent years, this has been challenged because friction coefficients do not reach the low values expected for fluid-film lubrication, any film would likely be on the same length scale as the cartilage roughness, and the soft and porous nature of cartilage which would not be able to sustain the pressures of a fluid film [6,29–31]. Theoretical arguments again full fluid films are convincing, especially in

combination with measurements of cartilage lubrication across the regimes of the Stribeck curve [6,29]. Still, direct visualization of the cartilage surface during sliding has not yet been accomplished. These tools are within reach. For example, the Bonassar and Cohen groups have used confocal elastography to visualize cartilage deformations under shear at the microscale [32]. Recently, Graham et al. reported on the development of a new tool to visualize fluid flow into cartilage during sliding by combining an in situ tribometer, confocal microscopy, and a fluorescent lubricant bath [33,34]. Developing tools and experiments to visualize fluid films at the cartilage surface would provide fundamental insights into cartilage lubrication, especially in the elastoviscous lubrication regime.

Decoupling Mechanics and Biology in Tribosupplementation: It is broadly believed that lubrication exerts a mechanobiological effect in the joint. Low friction prevents cartilage wear [10,11] and chondrocyte death [35–37]. Chondrocytes are essential to maintaining the cartilage extracellular matrix and their death has been linked to matrix degradation and the progression of OA [38]. As discussed in Chapter 1, tribosupplementation, or the injection of lubricin into the joint space is therapeutically beneficial in preventing the development of osteoarthritis. However, tribosupplementation has been performed in animal models where lubricin is observed to decrease in concentration after injury. This calls into question the applicability of tribosupplementation for cases where lubricin is observed to increase in disease or following traumatic joint injury.

Recent work has shown that lubricin acts an anti-inflammatory agent in the joint through its interactions with CD44 and TLR-4. Like HA, lubricin has been shown to bind to the CD44 receptor, a cell surface protein with many pro-inflammatory functions that is upregulated in OA and rheumatoid arthritis (RA) [39,40]. Exogenous lubricin has been found to suppress cytokine-induced proliferation of OA and RA synoviocytes as well as reduce the expression of catabolic

enzymes in arthritis like matrix metalloproteinases (MMPs) and pro-inflammatory cytokines [40,41]. Lubricin has also been shown to bind to toll-like-receptors -2 and -4 (TLR2 and TLR4) and block their activation [42,43]. TLRs are associated with the innate immune response, and in arthritis, they play a pro-inflammatory role in the joint by binding damaged matrix components and causing release of pro-inflammatory cytokines and activation of MMPs.

Therefore, the question remains as to how the mechanisms of action of a tribosupplement contribute to its disease modifying activity. Presently, these mechanisms of action seem divisible into two classes: mechanobiological (lubrication affecting chondrocyte health) and biological (suppressing pro-inflammatory pathways). Can these mechanisms be decoupled? Are the effects of each of these mechanisms additive or synergistic? Do these mechanisms vary with OA phenotype and synovial fluid composition? One could imagine comparing lubricin to a biologically inert, mimetic lubricin that replicates the lubricity of lubricin. Decoupling these mechanisms would prove challenging for several reasons, such as undiscovered biological pathways of lubricin or altered interactions with other synovial fluid components when using a mimetic (e.g., galectins, HA) that could confound outcomes. Though challenging, a better understanding of the mechanism of action would allow for the development of more effective and potentially safer therapies.

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