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BIOMARKERS OF KNEE JOINT HEALING IN ADOLESCENTS WITH ANTERIOR CRUCIATE LIGAMENT INJURIES

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GENERAL ABSTRACT

Objective: Anterior cruciate ligament (ACL) injuries are increasing in adolescents and increase the risk for early-onset knee osteoarthritis (OA). Biomarkers can be a non-invasive measure to assess physiological properties following knee injury or trauma. The objective of this thesis was to *i)* perform a systematic review to determine the most studied biomarkers of knee healing following ACL reconstruction (ACLR), and age of these patients, and *ii)* explore the feasibility of measuring these biomarkers in adolescents with ACL injuries.

Design: Studies were included if *i)* participants underwent ACLR, and *ii)* at least one biomarker of healing was measured. Participant age, sample(s) collected, and biomarker(s) studied were recorded. Interleukin-6 (IL-6), c-terminal crosslinking telopeptide of type II collagen (CTX-II) and procollagen type II collagen propeptide (PIICP) were then measured using ELISA in adolescents prior to ACLR in urine (*u*) and synovial fluid (*sf*). Spearman's Rho (r_s) coefficients were calculated to determine the association between *u*CTX-II/*sf*CTX-II, and *u*IL-6/*sf*IL-6. A ratio of PIICP: CTX-II was calculated to represent the ratio of cartilage synthesis to degradation.

Results: The review produced six studies evaluating healing following ACLR. IL-6 and CTX-II were the most studied (3/6 studies), and only one study included adolescents (age 19.6±4.5). Due to multiple undetectable biomarker levels, we could only report r_s for *u*CTX-II/*sf*CTX-II (r_s = -.200, *p*-value = .800, n=4). We also reported a ratio for *sf*PIICP: *sf*CTX-II (23.06 ±19.23).

Conclusion: Exploring biomarkers in adolescents was motivated by their unique physiology due to puberty, and this was the first study to do so. The findings from this pilot study indicate that further analysis is required to determine optimal sample preparation. This will allow for reliable results while studying the feasibility of these biomarkers during ACLR recovery. This insight can ensure more informed decision making by clinicians clearing patients for return-to-activity.

Key Words: Adolescents, Anterior Cruciate Ligament (ACL), Articular Cartilage Degradation, C-Terminal Crosslinking Telopeptide of Type II Collagen (CTX-II), Interleukin-6 (IL-6), Procollagen Type II Collagen Propeptide (PIICP)

CONTRIBUTIONS

Manuscript 1: Biomarkers of Knee Joint Healing Following Anterior Cruciate Ligament Reconstruction: A Systematic Review *(Presented as a podium presentation at the XXVIII Congress of the International Society of Biomechanics in 2021, and accepted as a podium presentation at Pediatric Research in Sports Medicine Society (PRiSM)9th Annual Meeting in 2022)*

The first manuscript of this thesis was a systematic review on biomarkers of knee joint healing following ACL reconstruction. The theoretical conception of this experiment was a concerted effort between L. Ek Orloff and M. Del Bel. Study design and search strategy was a combined effort of L. Ek Orloff, M. Del Bel, and K. Fournier. Study screening was performed by L. Ek Orloff and M. Del Bel. Finally, L. Ek Orloff was responsible for all analyses performed regarding this manuscript. The manuscript was written by L. Ek Orloff and edited by M. Del Bel, N. Romanchuk, S. Carsen, P. Imbeault, and D. Benoit.

Manuscript 2: Biomarkers of Knee Joint Healing in Adolescents with Anterior Cruciate Ligament Injuries *(Preliminary Results)*

The second manuscript of this thesis was guided by the results of manuscript 1, and measured biomarkers of knee joint healing in adolescents with ACL injuries. The theoretical conception of this experiment was conducted by L. Ek Orloff. Participant recruitment for this study was a combined effort of L. Ek Orloff, H. Livock, P. Sachsalber, S. Carsen, K. Kontio and A. Kerrigan. Data collection was a combined effort of L. Ek Orloff, S. Carsen, K. Kontio, A. Kerrigan, and N. Romanchuk. Analysis was performed by L. Ek Orloff and J.F. Mauger. Finally, L. Ek Orloff was responsible for all statistical analyses performed regarding this manuscript. The manuscript was written by L. Ek Orloff and edited by M. Del Bel, P. Imbeault, and D. Benoit.

LIST OF ACRONYMS

ACL	Anterior Cruciate Ligament
ACLR	Anterior Cruciate Ligament Reconstruction Surgery
BMI	Body Mass Index
CTX-II	C-Terminal Crosslinking Telopeptide of Type II Collagen
ELISA	Enzyme Linked Immunosorbent Assay
IL-6	Interleukin-6
KOA	Knee Osteoarthritis
OA	Osteoarthritis
PIICP	Procollagen Type II Collagen Propeptide
RTA	Return-To-Activity
SF	Synovial Fluid
U	Urine

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CHAPTER 1: RATIONALE FOR RESEARCH

Anterior cruciate ligament (ACL) injuries are increasing annually by 2.3% in adolescents (Beck et al., 2017) and lead to an increased risk for early onset knee osteoarthritis (OA), a degenerative joint disease (Guilak, 2011). This occurs due to increased inflammation and cartilage degradation in the knee brought on by abnormal loading patterns following injury, which are characteristics of knee OA (Andriacchi et al., 2004; Goldring, 2000, 2012).

Current ACL rehabilitation guidelines fail to consider the physiological healing process of the knee. Instead, current return-to-activity (RTA) criteria tends to focus on a patients' ability to meet functional bench marks of the entire lower body (Wright et al., 2015). Understanding how to measure this healing process could provide clinicians and physiotherapists with valuable prognostic insight while treating patients with ACL injuries and decrease their risk for further joint damage and early-onset knee OA.

Biomarkers could be used clinically to assess the healing process of the injury site. These include biomarkers of articular cartilage metabolism like c-terminal crosslinking telopeptide of type II collagen (CTX-II), a biomarker of articular cartilage degradation (Lohmander et al., 2003). Previous research has suggested that active growth plates may contribute to elevated concentrations of CTX-II in adolescents (Chmielewski et al., 2012). However, to the best of our knowledge, this has yet to be demonstrated. In addition, cytokines like interleukin-6 (IL-6) are upregulated following a joint injury and contribute to cartilage degradation (Higuchi et al., 2006; Pola et al., 2005), and have been identified as inhibitors of chondrogenesis (Friel et al., 2013). In this thesis, we will determine i) the most commonly studied biomarkers reflecting knee joint healing, and ii) the association between local synovial fluid and systemic urine levels of these biomarkers in adolescents with ACL injuries.

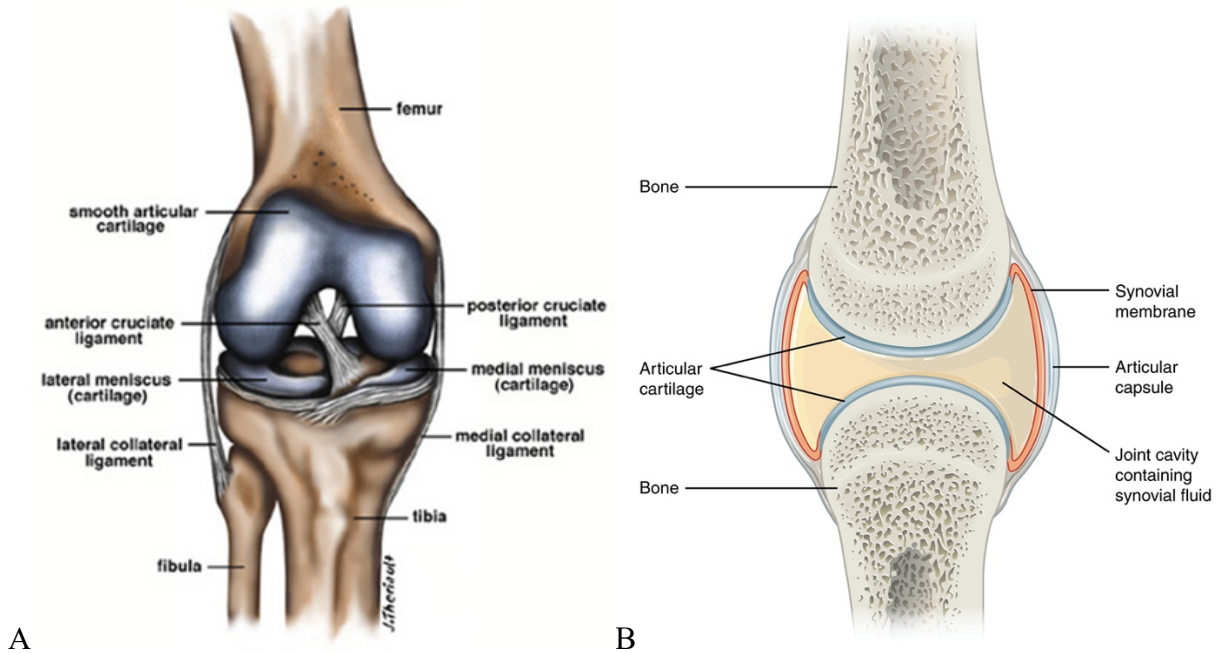
CHAPTER 2: LITERATURE REVIEW

2.1 The Anterior Cruciate Ligament

2.1.1 Anatomy

The knee joint is composed of osseous structures (distal femur, proximal tibia, patella), cartilage (meniscus, hyaline cartilage), four ligaments, and a synovial membrane (*Figure 2.1*; Mora et al., 2018). One of these ligaments is the ACL which extends from the anterior portion of the lateral femoral condyle to the medial posterior portion of the tibia (Duthon et al., 2006). It is an important component in facilitating knee joint function and stability and plays a role in resisting anterior translation and internal rotation of the tibia with respect to the femur (Frank R. Noyes, 2009; Papannagari et al., 2006). In addition, mechanoreceptors are found in several of the fibres in the ACL, which play an important role in providing proprioceptive feedback and contributes significantly to the functional stability of the knee (Johansson et al., 1991).

The ACL is made up of dense connective tissue and is in part comprised of type II collagen (Duthon et al., 2006), the primary component of cartilage. This collagen is found in the fibrocartilaginous tibial and femoral attachment sites of the ACL, in other parts of the ligament exposed to pressure and shear forces, and in the articular cartilage which covers the bones in the knee (Benjamin et al., 1990; Petersen et al., 1999).



A B

https://openstax.org/books/anatomy-and-physiology/pages/1-introduction

Figure 2.1. *Anatomical representation of the knee joint: 2.1.A. featuring knee ligaments, 2.1.B. featuring synovial membrane and joint cavity)*

2.1.2 ACL Injury Incidence and Mechanism

The ACL is the most frequently damaged knee ligament (Majewski et al., 2006) with ACL injuries increasing by 2.3% annually in adolescents between 1993-2013, in part due to increased participation in high-demand year-round sports at an earlier age (Beck et al., 2017). In addition, females are 2-8 times more likely to sustain an ACL injury than their male counterparts (Arendt et al., 1995; Beck et al., 2017; Micheli et al., 1999; Peck et al., 2013; Shaw et al., 2017). The majority (80%) of ACL injuries are non-contact in nature (Boden et al., 2000; Noyes et al., 1983), occurring during the deceleration of a jump landing, stop, or change in direction without direct contact from an another player or object (McLean.,1999).

2.1.3 Current ACL Rehabilitation and Return to Unrestricted Activity Criteria

Current RTA criteria following ACL injuries vary widely (Petersen et al., 2013), with functional tasks such as hopping tasks (Noyes et al., 1991) and isometric knee strength (Petersen

et al., 2014) as the prominent criteria. These functional tasks are used as an objective measure to track rehabilitation progress and eventual RTA status following an ACL reconstruction (ACLR; Davies et al., 2017; Myer et al., 2006). They are typically measured using a limb symmetry index (Abrams et al., 2014) and use a benchmark of at least 90% limb symmetry between the affected and unaffected limb to clear patients for RTA (Adams et al., 2012; Gokeler et al., 2017; Gokeler et al., 2017). The MOON protocol is an example of a commonly used rehabilitation protocol following ACLR consisting of rehabilitation guidelines based on functional criteria with a typical time frame of 5-6 months before clearing patients for RTA (Wright et al., 2015). However, it was recently demonstrated that passing current functional RTA criteria does not reduce the risk for ACL re-injury, which in turn increases a patient's risk for further joint damage and early-onset knee OA (Losciale et al., 2019; Webster et al., 2019). One clear limitation of current rehabilitation guidelines is the failure to consider a physiological reflection of healing through assessment of the metabolic activity in the knee joint. Returning to unrestricted activity too soon following ACLR, while the knee joint is still at a vulnerable stage in healing, could slow the healing process and increase the risk for further joint damage and early-onset knee OA (Chu et al., 2012; Friel et al., 2013). Identifying the ideal biomarkers of knee joint healing would provide the basis for future research targeted at providing insights for clinicians and physiotherapists responsible for creating and modifying RTA criteria and clearing patients for RTA.

2.2 Knee Osteoarthritis

2.2.1 Consequences of ACL injury

ACL injuries lead to an increased risk for early-onset knee osteoarthritis (OA; Guilak, 2011). The lack of a properly functioning ACL reduces the ability to resist excess anterior translation and internal rotation of the tibia with respect to the femur (Noyes et al., 2009;

Papannagari et al., 2006), and the proprioceptive abilities provided by the ACL in maintaining knee joint stability are significantly diminished (Johansson et al., 1991) resulting in abnormal mechanical loading patterns. This can cause increased cartilage degradation and inflammation in the joint, which are possible early signs of knee OA and principal characteristics of this degenerative joint disease (Andriacchi et al., 2004; Felson et al., 2000; Goldring, 2000, 2012; Noyes et al., 2009). Up to 62-80% of patients show radiographic signs of knee OA within 10-15 years following their initial ACL injury (Øiestad et al., 2010), and the risk for developing knee OA is increased by 6 fold in patients who have sustained an ACL injury compared to the uninjured population (Snoeker et al., 2020). The consequences of late-stage knee OA include structural joint change, functional limitations, and persistent pain, which can negatively impact quality of life (Stefan et al., 2007).

2.2.2 Mechanism from ACL injury to Knee OA

Knee OA is a degenerative joint disease primarily characterized by the progressive loss of articular cartilage which covers the osseous structures in the knee (Hsu et al., 2020). The mechanism of cartilage breakdown and progression to OA following an ACL injury is multifactorial (Friel et al., 2013), but may be in part due to the initial injury to the subchondral bone and cartilage. At the time of injury, the high force of the trauma disrupts the intra-articular structures (Roos et al., 1994). Bone bruises occur in 80–90% of patients with an acute ACL injury (Nishimori et al., 2008), most commonly on the posterolateral tibial plateau and the anterolateral femoral condyle. These lesions suggest that articular cartilage sustains a considerable mechanical impact at the time of injury (Potter et al., 2012).

In addition to the initial trauma, the lack of an intact ACL leads to chronic changes in the mechanical loading patterns of the knee and increases forces on the cartilage and other joint

structures (Andriacchi et al., 2004). Herzog et al. reported that the lack of an ACL leads to a thicker, softer and more permeable articular cartilage (Herzog et al., 2004). Articular cartilage in synovial fluid retains its integrity and function through regular and consistent loading patterns. Under-loading (which can occur following a knee injury), over-loading, or changing the stability of the joint along with poor muscle control and weakness can cause the joint to adapt to these new and altered loading patterns and lead to loss of articular cartilage, as demonstrated in animal models (Herzog et al., 2004; Moskowitz et al., 1979). Therefore, subsequent intra-articular injuries occur over time, especially to the articular cartilage. These lesions play a role in the development of joint damage and knee OA, as revealed in multiple studies that reported higher rates of OA in patients with concomitant intra-articular injuries (Friel et al., 2013). Finally, despite the intention of preventing further damage by increasing the biomechanical stability of the knee joint, ACL reconstruction can further alter knee kinematics and therefore fail to restore proper mechanical loading in the knee (Papannagari et al., 2006). The incidence of knee OA following ACL reconstruction remains as high as 87% (Nakata et al., 2008; Shelbourne et al., 1997), and the risk of developing knee OA increases by 6 fold in compared to the general population once an ACL injury has been sustained (Snoeker et al., 2020).

2.2.3. Assessments of Knee Health

The assessment of OA relies on radiography (Kellgren et al., 1957) where radiographs are scored on a grading scale from 0-4, correlating with increased severity of OA (Kohn et al., 2016). This radiographic assessment of knee OA is the gold standard, but it is limited in its inability to measure early articular cartilage degradation and loss and cannot be used as a non-invasive early detection tool for signs indicating increased risk for knee OA (Tabassi et al., 2007). In recent years, biological markers have been used as an objective measure to indicate

cellular biochemical function (Biomarkers Definitions Working Group., 2001; Strimbu et al., 2010). Biomarkers can be measured in synovial fluid, serum, plasma, urine, and other fluids and tissues, and therefore have the potential to be a non-invasive measure able to assess physiological properties of the knee joint's healing progress. Identifying abnormal levels of metabolic by-products, and inflammation, which are indicators of increased risk for knee OA, can be achieved through measures of specific biomarkers reflective of these processes in the knee joint (Garnero et al., 2001; Nguyen et al., 2017).

2.3 Characteristics of Knee OA

2.3.1 Articular Cartilage Degradation

Articular cartilage, the primarily affected structure in knee OA, protects the bone below it and serves as a wear-resistant, friction-reducing surface with highly compressive stiffness (Hussain et al., 2016). This structure evenly distributes forces onto the bones below it in the knee joint (Hu et al., 2003). It is a non-vascularized hyaline cartilage and specialized connective tissue composed of two main components: chondrocyte cells and extracellular matrix (ECM) made up of water, proteoglycans (Hussain et al., 2016), and type II collagen providing tensile strength (Buckwalter et al., 1998; Fox et al., 2009). Chondrocytes are highly specialized metabolically active cells involved in the synthesis, degradation, maintenance and repair of the cartilage's ECM using degradative enzymes, and are sparsely distributed throughout the ECM (Archer et al., 2003; Buckwalter et al., 1998; Fox et al., 2009). These cells are vital for proper articular cartilage maintenance and function through chondrogenesis (Goldring, 2012). Chondrocytes detect an imbalance in the ECM composition and organization and synthesize new molecules to restore proper macromolecule levels. This ensures the preservation of a healthy structure serving

as the wear-resistant articular cartilage surface protecting the bones in the knee joint (Hu et al., 2003).

The macromolecules of the ECM are constantly lost through degradation which occurs naturally through load bearing of the joint during normal daily activities, like walking. In a healthy knee there is enough articular cartilage synthesis to counteract the natural degradation and the metabolic cycle continues at a constant and stable rate (Buckwalter et al., 1998). Normal articular cartilage metabolism occurs when the rate of cartilage degradation is in homeostasis with the rate of cartilage synthesis (Buckwalter et al., 1998). An increase in articular cartilage degradation is a principal characteristic of knee OA, but a certain level of degradation is normal when synthesis can keep up. Abnormal metabolism can be induced by an ACL injury as the resulting joint instability acts as a stressor on the knee and leads to abnormal mechanical loading and articular cartilage damage, as previously detailed in section *2.2.1*. This increased articular cartilage damage is a new abnormal stressor, causing this cycle of joint damage to continue if proper and timely intervention is not implemented (Guilak, 2011). Once the ACL is torn, the knee joint is at risk for internal damage stemming both from the initial injury and the subsequent adjustment as the stability and proprioception provided by the ACL is not absent. This leads to altered mechanical loading in the knee joint, leading to degradation on parts of articular cartilage in the knee.

2.3.1.1 Biomarkers of Articular Cartilage Metabolism *(Degradation and Synthesis)*

Biomarkers of cartilage metabolism known to reflect disease severity in the knee include those indicating both cartilage degradation and synthesis. Collagen Type II Cleavage (C2C), Type II Collagen Cleavage Neopeptide (C2; Conrozier et al., 2008), C-Terminal Crosslinking Telopeptide of Type II Collagen (CTX-II; Kalaï et al., 2012), Cartilage Oligomeric Matrix

Protein (COMP; Verma et al., 2013), are all markers of cartilage degradation. Synthesis of Type II Procollagen (CPII) and N-Propeptide of Collagen IIA (PIIANP) are examples of biomarkers of cartilage synthesis (Conrozier et al., 2008).

2.3.2 Inflammation in the Knee Joint

Articular cartilage degradation is not alone in characterizing knee OA, as symptoms and progression of cartilage degeneration have been associated with synovitis which is the inflammation of synovial tissue (Ayral et al., 2005; Hill et al., 2007). A knee injury, like an ACL injury, will alter levels of synovial fluid which contains compounds some of which contribute to joint degeneration (Friel et al., 2013). Studies have demonstrated that chondrocytes, osteoblasts and inflamed synovium all produce and contribute to various pro-inflammatory cytokines (Haringman et al., 2004). Cytokines have also been reported to increase cartilage degradation (Dinarello, 2000, 2007; Orita et al., 2011; Sorsa et al., 2004). Soluble mediators like cytokines and prostaglandins stimulate chondrocytes to produce matrix metalloproteinases, responsible for the degradation of the extracellular matrix in cartilage metabolism (Sorsa et al., 2004).

In the knee joint, the synovial membrane is responsible for the production of the synovial fluid, which provides lubrication and nutrients to the avascular cartilage in the joint (Sharma et al., 2017). Inflammation in OA is often associated with low-grade synovitis (Pessler et al., 2008) where synovial tissues secrete cytokines and causes immune cell concentrations to increase (Scanzello et al., 2008). Cytokines are soluble factors produced by cells of the immune system in response to infection, inflammation or trauma, like an ACL injury or reconstruction surgery (Dinarello, 2000, 2007; Orita et al., 2011; Sorsa et al., 2004).

2.3.2.2 Biomarkers of Inflammation

Multiple inflammatory cytokine biomarkers, like tumor necrosis factor-alpha, Interleukin-1 (IL-1β), and Interleukin-6 (IL-6), are upregulated following a joint injury and have been shown to contribute to the cartilage degradation process (Cuellar et al., 2010; Goldring, 1999; Higuchi et al., 2006; Pola et al., 2005) and inhibit chondrogenesis (Friel et al., 2013). These studies show consistently that cytokines are elevated immediately following injury and likely persist for a prolonged period of up to 10-15 years. Furthermore, ACL reconstruction surgery is an additional trauma to the joint resulting in prolonged joint inflammation and postoperative hemarthrosis (Jawa et al., 2011b).

2.4 Physiology of Puberty

ACL injuries in adolescents are unique compared to those suffered by adults, meaning that research on biomarkers of healing following ACL injuries cannot simply be extrapolated from adults to this younger, growing population. This discrepancy is due to puberty and its associated hormone changes, and growth plate activity and fusion.

2.4.1 Growth Plate Activity and Fusion

Endochondral bone development is the process through which all bones grow to generate the skeleton (Kronenberg, 2003). The epiphyseal plate, more commonly referred to as the growth plate, is a cartilaginous structure which drives the growth of long bones in length through chondrogenesis and is responsible for growth in height, especially notable during adolescence (Lui et al., 2014; Shim, 2015). In long bones, like the femur and tibia (Martínez et al., 2007), the cartilage called the growth plate consists of chondrocytes which proliferate between regions of bone of the primary and secondary ossification centres (Kronenberg, 2003). Growth plates are uniquely present in the pediatric and adolescent populations, and disappear through fusion following adolescence (Kronenberg, 2003). Tanner Stages are a measure of classifying

adolescents into 5 stages of puberty with stage 1 indicating the onset of puberty and stage 5 the final adult form (Emmanuel et al., 2018). Thus, Tanner stages can be used as an approximate measure of skeletal maturity due to the correlation between progression through puberty and growth plate fusion.

2.4.2 Effect of Growth Plates on Cartilage Metabolism

Longitudinal bone growth is primarily mediated by growth hormone (GH) and insulin-like growth factor-I (IGF-I). During puberty, there is a 1.5- to 3-fold increase in the pulsatile secretion of GH and a more than 3-fold increase in the concentration of serum IGF-I (Albin et al., 2012). IGF-I circulates and is found locally at the epiphyseal plate, where it is produced by the chondrocytes. Here, it plays an important role in the differentiation, proliferation and hypertrophy of chondrocytes, the production of extracellular matrix, and ossification in the epiphyseal plate (Giustina et al., 2015).

GH and estrogen are closely linked in the regulation of growth and pubertal development as estrogen induces the stimulation of the GH-IGF-I axis and a pubertal growth spurt during adolescence. The binding of estrogen to its receptors in the growth plate is suspected to be related to the pubertal growth spurt and epiphyseal fusion, with some research suggesting that epiphyseal fusion occurs when the proliferative capacity of growth plate chondrocytes, influenced by estrogen, is exhausted (Chagin et al., 2007; Juul, 2001; Weise et al., 2001). Through stimulation of the GH-IGF-I axis, high levels of estrogen stimulate secondary sexual characteristics and epiphyseal fusion in late puberty (Juul, 2001).

This endochondral bone formation process primarily involves highly active chondrocytes and the secretion of type II collagen at the growth plates in the long bones of the leg. This secretion of type II collagen can be notable in levels of CTX-II in adolescents (Chmielewski et

al., 2012), and underlines the possibility of active growth plates in adolescents to interfere with the ability of CTX-II to reflect the healing process of the knee joint when growth plates are active. The ability of cytokines to stimulate chondrocytes and cartilage degradation (Sorsa et al., 2004) adds to the uncertainty of how cytokine biomarkers are impacted by the increase in growth plate activity and the altered physiological environment uniquely present in adolescents. In fact, as revealed through our review (Ek Orloff, *under review*) these measures are yet to be reported in a population made up solely of adolescents.

2.5 Gap in Knowledge

Research on adolescents with ACL injuries is necessary to build a scientific foundation on biomarkers reflecting the healing process of the knee following injury and reconstruction surgery. This knowledge could provide basis to study the feasibility of these biomarkers longitudinally during rehabilitation. As a result, more informed and accurate decision making can be made by clinicians and physiotherapists while treating their patients recovering from ACL reconstruction. This could reduce their risk for early-onset knee OA later in life by re-introducing increased mechanical loading to the knee joint when most appropriate based on the internal healing progress.

CHAPTER 3: PURPOSE AND HYPOTHESIS

The purpose of this thesis was to *i)* perform a systematic review of existing literature to determine the most commonly studied biomarkers of knee joint healing following ACL injuries, and *ii)* determine the association between local synovial fluid and systemic urine biomarkers of knee joint healing (IL-6, CTX-II, CTX-II: PIICP) in adolescents with ACL injuries. To achieve this objective, two research questions were posed:

Question 1) *Based on existing literature, which biomarkers are the most commonly used when studying knee joint healing in patients with ACL injuries, and what is the age of these study participants?*

Previous research suggests cytokines are up to 15 times more elevated in acute compared to chronic ACL injuries (Bigoni et al., 2016). IL-6 is overwhelmingly included in cytokine panels following ACL injuries and is an important biomarker contributing to correlations with cartilage degradation during joint injury and healing, and its correlation with other cytokines during this process (Bigoni et al., 2016; Friel et al., 2013; Lattermann et al., 2018; Wang et al., 2020). Biomarkers of articular cartilage degradation have also shown promising results as prognostic and diagnostic tools for knee OA and joint health (Bellido et al., 2011; Delmas, 2009). Following ACL injuries, a cartilage degradation biomarker (*u*CTX-II) has correlated moderately (r=0.4) with knee joint pain and function (Chmielewski et al., 2012), and biomarker panels repeatedly indicate *u*CTX-II as a reoccurring and consistent prognostic biomarker in adults (Lotz et al., 2013). This leads us to hypothesize that CTX-II and IL-6 will be the most common biomarkers used to assess healing *(H1a).*

A previous systematic review on osteoarthritis-related biomarkers summarized ages of participants in their included studies (Harkey et al., 2015). All study participants were adults,

leading us to hypothesize that participant ages in the included studies for this systematic review will also be adult *(H1b).*

Question 2) *What is the association between CTX-II, IL-6 and PIIC levels measured in urine and synovial fluid?*

Previous research has reported increased concentrations of CTX-II in adults with ACL injuries compared to uninjured controls in both urinary and synovial fluid (Chmielewski et al., 2012; Lohmander et al., 2003). Research conducted with adults has demonstrated elevated levels of *u*CTX-II, which correlate (r=0.309-0.622) with knee OA disease severity (Garnero et al., 2001; Jordan et al., 2006; Tanishi et al., 2009). Levels of articular cartilage synthesis have remained statistically significantly elevated relative to baseline at 12 and 24-months following ACLR (Beynnon et al., 2005) Thus, we hypothesize there will be a moderate correlation (0.4-0.7) between *sf* and *u*CTX-II, and PIICP: CTX-II levels in adolescent patients with ACL injuries *(H2a and H2b, respectively)*.

Levels of *u*IL-6 (Park et al., 2016; r=0.2) and *sf*IL-6 (Hayward et al., 2011; Higuchi et al., 2006; r=0.723) have both been correlated with levels of joint damage in arthritic diseases and knee inflammation following ACL injuries in separate studies. Additionally, Anil et al. reported a statistically significant increase in the concentrations of IL-6 ($p = 0.014$), between the time of surgery and a mean of 10.4 days following ACLR, reflecting the early healing phase in the knee (Anil et al., 2019). However, this correlation remains to be explored in patients with ACL injuries. Thus, we hypothesize a moderate correlation (0.4-0.7) between *u*IL-6 and *sf*-IL6 *(H2c)*.

CHAPTER 4A: METHODOLOGY: *Study One: Systematic Review*

4A.1 Study Design

This systematic review was conducted in collaboration with the University of Ottawa library resources and librarian Karine Fournier. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines was followed when preparing, conducting and reporting this systematic review (Moher et al., 2009).

4A.2 Protocol

4A.2.1 Search Strategy

The search engine Ovid was used and the databases Medline, Embase, SCOPUS and Web of Science were searched through September 2nd, 2020 to identify studies reporting biomarkers used to assess knee joint health and healing in patients with ACL injuries. Search terms consisted of: Concept 1: Anterior Cruciate Ligament, and Concept 2: Biomarkers, as is outlined in further detail for each database in *Appendix I (Table A.1.)* Terms within each concept were combined with the OR Boolean operator, and the two concepts were combined with the AND Boolean operator. Publication details from all identified studies were exported to Covidence systematic review software (Veritas Health Innovation, Melbourne, Australia; www.covidence.org) and duplicates were removed.

4A.2.2 Study Identification

Two authors (L.E.O. and M.D.B.) independently reviewed the title and abstract of each article identified through the literature search. The full text of an article was obtained and evaluated when eligibility could not be assessed from the first screening. Full text screening was performed independently by two authors (L.E.O. and M.D.B.). Any discrepancies were resolved

through a consensus discussion between reviewers, and a senior author (D.L.B.) was consulted if a disagreement cannot be resolved.

Studies were included if they: (1) include participants who have sustained a primary ACL injury, (2) measured at least one biomarker of knee joint healing at more than one time point, and (3) published in either English or French. Grey literature including conference proceedings, letters to editors, case reports, clinical commentaries, pilot studies, and review articles were excluded.

4A.3 Data Analysis

4A.3.1 Assessment of Study Quality

The NIH quality assessment tool (before-after (pre-post) study with no control group) was used to assess the quality of the final included papers. Each study was independently assessed by two authors (L.E.O. and M.D.B.), and any disagreements were discussed until a consensus is reached.

4A.3.2 Data Extraction and Outcome Measures

A standardized data sheet was used to record the following information and outcome data: sample size; patient's age; time between injury/surgery and sample collection; biological sample(s) collected; biomarker(s) analyzed. One author (L.E.O.) recorded all of the pertinent data from the included articles, and other author (M.D.B.) independently reviewed these data for accuracy and completeness. The primary outcome of interest was the biomarkers reflecting knee joint healing in patients with ACL injuries according to: i) biomarker(s) measured, and ii) biological tissue(s) collected for this analysis. The secondary outcome of interest was the iii) age of participants enrolled in the included studies. Additional outcomes of interest included the

relationship between levels of different biomarkers measured, and the levels of these biomarkers between participants with ACL injuries and control groups, where applicable.

CHAPTER 4B: METHODOLOGY: *Study Two*

4B.1 Study Design

This methodology section pertains to studies two and three as stated in *Chapter 3*. The biological sample collection of urine took at the home of participants the day before each ACL reconstruction surgery. Synovial fluid samples were collected at CHEO at the time of surgery performed by Dr. Sasha Carsen or his colleague Dr. Ken Kontio; both orthopedic pediatric surgeons at CHEO. Samples analyses were performed at the University of Ottawa under the supervision of Dr. Pascal Imbeault.

4B.2 Participants

A priori power analysis in G*Power software (3.1.0, Dusseldorf, Germany) was performed based on studies on local and systemic levels of articular cartilage degradation and inflammation biomarkers reflecting knee joint health in adults (Higuchi et al., 2006; Lohmander et al., 2003) or animal models (Coyle et al., 2012) with ACL injuries. This established a sample size (n) of 19 as required in order to achieve a power of 0.8 with an input correlation of 0.6 at $\alpha = 0.05$ (Coyle et al., 2012; Higuchi et al., 2006; Lohmander et al., 2003). As a buffer, twenty-five patients with ACL injuries between the ages of 10-18 years were recruited from CHEO as participants. Inclusion criteria required participation in organized sports, defined as current competitive physical activity with an official or judge for uninjured participants (Fabricant et al., 2013), and active participation at the time of injury. Participants did not have: i) a history of an arthritic condition (i.e. osteoarthrosis, rheumatoid arthritis), ii) a history of previous traumatic lower-limb or knee injury (i.e. meniscal tear, ligament rupture, broken bones), iii) a history of any inflammatory disease or immunocompromised (Lattermann. et al., 2016), or iv) prolonged steroid use.

4B.3 Protocol

4B.3.1 Consent Form and Questionnaires

Prior to data collections all participants read and verbally consented based on a verbal consent form approved by the CHEO Research Institute Ethics Board and the University of Ottawa Research Ethics Board. Participants completed the following questionnaires; i) an assessment of sport exposure (Pedi-FABS) (Del Bel et al., 2020; Fabricant et al., 2013), ii) a subjective assessment of knee joint function (Pedi-IKDC; Kocher et al., 2011), iii) a pubescent-stage self-assessment form (Tanner; Taylor et al., 2001), iv) a subjective assessment of activity level (Tegner activity scale; Tegner et al., 1985); and v) an assessment of their psychological readiness to return to sport following ACL injury and reconstruction surgery (ACL-RSI; Webster et al., 2008). Participants completed these forms virtually through a research survey distribution platform called RedCap.

4B.3.2 Participant Preparation

Participants refrained from any high-impact activity in the 24-hours prior to biological sample collection to avoid influencing CTX-II levels (O'Kane et al., 2006). Participants were mailed a sterile specimen container, ice pack, and instruction sheet on how to collect, store and transport their own urine sample. Second void urine sample collection occurred the day before surgery (Chmielewski et al., 2012; Cibere et al., 2009; Dam et al., 2009; Huebner et al., 2014; Kalaï et al., 2012; Xin et al., 2017) at a consistent window between 12:00pm and 4:00pm (Gordon et al., 2008; Kong et al., 2006). Patients arrived at CHEO for their scheduled ACL reconstruction surgery and synovial fluid samples were collected at the time of their surgery.

4B.3.3 Urine Sample Collection Protocol and Materials

Urine samples were brought to CHEO by the participant and collected upon their arrival to the pre-op area. They were immediately labelled with the date, project code, participant code and contents and temporarily stored on ice. A permit for the transportation of dangerous goods (Classes 6.2 and 9) was received from the CHEO Research Institute and samples were immediately transported by car to the physiology lab of Dr. Pascal Imbeault. Samples were centrifuged at 3000 rpm for 20 minutes and the supernatant was aliquoted and stored in duplicates at -80 °C awaiting analysis.

4B.3.4 Synovial Fluid Sample Collection Protocol and Materials

On the day of their ACL reconstruction surgery, participants followed regular pre-operative protocol and were put under anesthesia. They then had their affected knee joint aspirated of synovial fluid to dryness with a syringe in the operating room and the obtained volume noted. The synovial fluid sample were immediately transferred from the syringe to a test tube (Lohmander et al., 2003) labelled with the date, project code, participant code and contents, and stored on ice for transportation by car for storage as mentioned in *4B. 3. 3*. The samples were centrifuged at 3000 rpm for 20 minutes and the supernatant was aliquoted and stored in duplicates at -80 °C awaiting analysis.

4B.4 Data Analysis Enzyme Linked Immunosorbent Assay

Urine and synovial fluid samples were retrieved and measured at 450 nm using the Synergy HT Spectrophotometer Plate Reader (BioTek Instruments Inc., Winooski, VT, USA). Biomarker concentrations were measured using ELISA kits for levels of CTX-II (MyBioSource Inc, San Diego, California, USA), PIICP (MyBioSource Inc, San Diego, California, USA), IL-6 (Abcam Inc, Toronto, ON). Urine samples were analyzed for levels of creatinine and urinary biomarkers of CTX-II, PIICP, and IL-6 was corrected for creatinine to adjust for variable urine

dilution (Cayman Chemical Company, Ann Arbor, MI, USA; Chmielewski et al., 2012). The concentration of creatinine (mmol/L) was determined using Enzyme Linked Immunosorbent Assay (Cayman Chemical Company, Ann Arbor, MI, USA).

4B.4.2 Statistical Analysis

Two continuous variables; the concentrations of the local synovial fluid biomarker (*sf*CTX-II, or *sf*IL-6) and its biomarker matched systemic urinary biomarker (*u*CTX-II, or *u*IL-6), were used in this analysis. The null hypothesis for this statistical test was that there is no correlation between the concentrations of the local and systemic of each biomarker pair in adolescents with ACL injuries. A scatterplot of both variables was visually inspected for a linear relationship and outliers. A Shapiro-Wilk test was performed in order to evaluate the assumption of normality. If the data violated the assumption of normality a transformation (e.g. logarithmic transform) was performed (Chmielewski et al., 2012; L. Stefan Lohmander et al., 2003; Sowers et al., 2009). The Spearman's Rho (r_2) correlation coefficient was determined using the concentration of the local synovial fluid biomarker in pg/mL and the creatinine-corrected concentration of systemic urinary in μmol/l (Chmielewski et al., 2012). The Spearman's Rho (r_2) correlation coefficient was then extracted in order to determine the strength of association of the coefficient value with a strong correlation >0.7, moderate, 0.4-0.7, and low <0.4. A significance level of $p<0.05$ was used for all statistical tests, and statistical analysis was performed using SPSS software version 17.0 (IBM, Chicago, IL, USA).

CHAPTER 5: MANUSCRIPT 1

Biomarkers of Knee Joint Healing Following Anterior Cruciate Ligament Reconstruction: A Systematic Review

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Abstract

Objective: Anterior cruciate ligament (ACL) injuries have been increasing, especially amongst adolescents. These injuries can increase the risk for early-onset knee osteoarthritis (OA). In recent years, biological markers have been used as an objective measure to indicate cellular biochemical function. Biomarkers can be a non-invasive measure to assess physiological properties of the knee following injury, trauma, or disease like knee OA. The purpose of this review was to identify commonly studied biomarkers assessing knee joint healing in patients following ACL reconstruction (ACLR) and identify the age of these study participants.

Design: Following PRISMA guidelines, Medline, Embase, SCOPUS and Web of Sciences were searched up until September 2nd, 2020. Studies were included if they *i)* included participants who had sustained a primary ACL injury and undergone a subsequent ACLR, and *ii)* measured at least one biomarker of healing at multiple time points. The primary outcome variables were participant age; biological sample(s); biomarker(s) analyzed.

Results: Six studies evaluated healing following ACLR. IL-6 and CTX-II were the most common biomarkers reflecting joint healing (3/6 studies). One study evaluated healing in adolescents (age 19.6±4.5), reporting a negative correlation between age and CTX-II levels ($r=-.769$, $p < 0.001$).

Conclusions: Considering that adolescents are unique from adults due to anatomical and physiological changes that occur during puberty, future research should target an adolescent-specific population. In addition, this research should explore IL-6 in synovial fluid and CTX-II in urine, which we identified as the most relevant biomarkers of healing. Finally, we propose that future research should investigate how biomarkers of healing can be incorporated into return-to-activity (RTA) assessment following ACLR in adolescents to improve outcomes and reduce the risk of early-onset knee OA.

Key Words: Adolescents, Anterior Cruciate Ligament (ACL), Articular Cartilage Degradation, C-Terminal Crosslinking Telopeptide of Type II Collagen (CTX-II), Interleukin-6 (IL-6)

Introduction

The anterior cruciate ligament (ACL) is the most frequently damaged ligament in the knee joint complex (Majewski et al., 2006), with an increasing prevalence of 2.3% annually in adolescents between 1993-2013 (Beck et al., 2017). ACL injuries have also been associated with an increased risk for early-onset knee osteoarthritis (OA; Guilak, 2011). In fact, up to 62-80% of patients show radiographic signs of knee OA within 10-15 years following their initial ACL injury (Øiestad et al., 2010). Although the work by Øiestad et al. (2010) was focused on adults, these results should not be disregarded for adolescents, until a similar longitudinal study in this cohort is completed. For context, if an adolescent (< 18 years old) sustains an ACL injury, and the same risk of early-onset knee OA exists in this population, these patients are likely to have symptoms of knee OA before the ages of 30-35.

The progression to OA following an ACL injury is multifactorial (Friel et al., 2013), but is often due to abnormal mechanical loading patterns causing an increase in cartilage degradation and inflammation in the joint (Andriacchi et al., 2004; Felson et al., 2000; Goldring, 2000, 2012; Noyes et al., 2009). During the initial injury, the high force of the trauma disrupts the intra-articular structures which suggests that articular cartilage sustains a considerable mechanical impact at the time of injury (Potter et al., 2012; Roos et al., 1994). In addition, the lack of a functioning ACL reduces the ability to resist excess anterior translation and internal rotation of the tibia with respect to the femur (Noyes et al., 2009; Papannagari et al., 2006), and diminishes the proprioceptive feedback required for maintaining knee joint stability (Johansson et al., 1991). Finally, although ACL reconstructions are intended to restore the biomechanical stability of the knee joint and prevent further damage, some research suggests that knee kinematics and proper mechanical loading in the knee joint are not restored following surgery (Papannagari et al.,

2006). Thus, both the initial ACL injury and the subsequent reconstruction can lead to abnormal joint loading patterns and OA progression. Consequences of late-stage knee OA include structural joint damage, functional limitations and persistent pain which negatively quality of life (Stefan et al., 2007).

ACL injuries are multifaceted in nature, with biomechanical, physiological and psychosocial components (Kvist et al., 2018); thus, rehabilitation and return-to-activity (RTA) criteria aimed at reducing re-injuries should take the same multifaceted approach. Current RTA criteria following ACL injuries vary widely (Petersen et al., 2013) consisting primarily of functional tasks such as single-limb hops (Noyes et al., 1991) and isometric strength (Petersen et al., 2014). However, it has recently been reported that passing current RTA criteria does not reduce the risk for ACL re-injury (Losciale et al., 2019; Webster et al., 2019), which in turn increases a patient's risk for further joint damage and development of early-onset knee OA (Losciale et al., 2019; Webster et al., 2019). Returning to unrestricted activity too early, while the knee joint is still at a vulnerable stage in healing, slows the healing process and increases the risk for knee OA (Chu et al., 2012; Friel et al., 2013). One clear limitation of current rehabilitation guidelines is the failure to consider a physiological reflection of healing through assessment of the metabolic activity in the knee joint. As the incidence of knee OA following ACL reconstruction remains as high as 87% (Nakata et al., 2008; Shelbourne et al., 1997), biomarkers of healing could provide insight regarding when it is most appropriate to introduce an increased mechanical loading to the healing knee joint.

Biomarkers are used as objective measures of cellular biochemical function, and can be analyzed in serum or synovial fluid, and through non-invasive biological samples such as urine and saliva (Biomarkers Definitions Working Group., 2001; Strimbu et al., 2010). Analyses of

biomarkers allows assessments of physiological properties of the knee joint's healing process by measuring, for example, the by-products of the metabolic processes of bone and cartilage (a potential indicator of knee OA risk; Garnero et al., 2001; Nguyen et al., 2017). It is important to note that research on biomarkers of healing following ACL injuries cannot be extrapolated from adults to adolescents. This discrepancy is due to growth plate activity and fusion, and hormone changes which occur during puberty. The epiphyseal plate, more commonly referred to as the growth plate, is a cartilaginous structure which drives the growth of long bones through chondrogenesis and is responsible for growth in height, especially notable during adolescence (Liu et al., 2014; Shim, 2015). In long bones, like the femur and tibia (Martínez et al., 2007b), the growth plate consists of chondrocytes which are responsible for articular cartilage metabolism (Kronenberg, 2003b). Thus, adolescents have a normal and necessary source of cartilage metabolism at their unfused growth plates, which becomes a major factors when looking at cartilage metabolism as markers for joint damage and disease prevention (Kronenberg, 2003b).

Given that the rates of adolescent ACL injuries are on the rise (Beck et al., 2017), and that adolescents have unique physiology from adults, research on physiological measures of knee joint healing should be adolescent-specific. In order to determine which physiological measures to study in adolescents, this systematic review explored the most utilized biomarkers reflecting knee joint healing in patients with ACL injuries, as well as the age of the included study participants. We hypothesize that IL-6 and CTX-II will be the most prevalent biomarkers used to assess knee joint healing. IL-6 is overwhelmingly included in research on cytokine panels following ACL injuries and is an important biomarker associated with cartilage degradation during joint injury and healing, as well as with other cytokines during this process (Bigoni et al.,

2016; Cuellar et al., 2018; Chu et al., 2013; Wang et al., 2020). In addition, cartilage degradation biomarker (*u*CTX-II) is moderately correlated (r=0.4) with knee joint pain and function (Chmielewski et al., 2012), and biomarker panels repeatedly indicate *u*CTX-II as a reoccurring and consistent prognostic biomarker in adults (Lotz et al., 2013). A previous systematic review on osteoarthritis-related biomarkers summarized ages of participants in their included studies (Harkey et al., 2015). All study participants were adults, leading us to hypothesize that participant ages in the included studies for this systematic review will also be adults.

Methods

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed when preparing, conducting and reporting this systematic review (Moher et al., 2009).

The Medline, Embase, SCOPUS and Web of Science databases were searched up until September 2nd, 2020. Studies which reported using biomarkers to assess knee joint health and healing in patients with ACL injuries were identified. A university librarian assisted with the creation and execution of the search strategy (Table 5.1).

Table 5.1
Search strategies for each database that were used to compile articles for screening in study 1.

	SEARCH ALGORITHMS			
	MEDLINE - OVID	**EMBASE - OVID**	**Web of Science**	**SCOPUS**
Concept 1 Subject Heading	exp Anterior Cruciate Ligament Reconstruction/	exp Anterior Cruciate Ligament Reconstruction/	-- Anterior Cruciate Ligament Reconstruction/	-- Anterior Cruciate Ligament Reconstruction/
Concept 1 Key Word Search	*((ACL or anterior cruciate ligament) adj4 (injur* or reconstruction* or repair* or graft* or surger* or operat*)).ti,ab,kf.*	*((ACL or anterior cruciate ligament) adj4 (injur* or reconstruction* or repair* or graft* or surger* or operat*)).ti,ab,kw.*	*TS=ACL or anterior cruciate ligament NEAR/4 (reconstruction* or repair* or graft* or surger* or operat*)*	*TITLE-ABS-KEY ((ACL or anterior cruciate ligament) W/4 (injur* reconstruction* or repair* or graft* or surger* or operat*))*
Concept 1 (final)	1 or 2	1 or 2	1 or 2	S1 or S2
Concept 2 Subject Headings	Biomarker/ Biological Marker	Biomarker/ Biological Marker	Biomarker OR Biological Marker	Biomarker OR Biological Marker
Concept 2 Key Word Search	*(bio* adj2 (mark* adj 4 (cartilag* or bone or inflam* or knee adj3 (heal*)))).ti,ab,kf.*	*(bio* adj3 (mark* adj4 (cartilage* or bone* or inflam* or knee adj3 (heal*)))).ti,ab,kw.*	*TS=bio* NEAR/3 (mark* NEAR/4 (cartilage* or bone* or inflam* or knee NEAR/3 (heal*)))*	*TITLE-ABS-KEY (bio* W/3 (mark* W/4 (cartilage* or bone* or inflam* or knee W/3 (heal*))))*
Concept 2 (Final)	3 or 4	4 or 5	3 or 4	S3 or S4
Language	English or French	English or French	English or French	English or French
Years	2000-2021	2000-2021	2000-2021	2000-2021
Final search *Date Search Executed*	5 and 6 September 2nd, 2020	3 and 6 September 2nd, 2020	5 and 6 September 2nd, 2020	S5 AND S6 September 2nd, 2020

Where possible, terms were mapped to medical subject headings and searched using keywords. Publication details from all identified studies were exported to Covidence systematic review software (Veritas Health Innovation, Melbourne, Australia; www.covidence.org) and duplicates were removed. Two authors (L.E.O. and M.D.B.) independently reviewed the title and abstract of each article identified through the literature search. The full text of an article was obtained and evaluated when eligibility could not be assessed from the first screening. Full text screening was performed independently by two authors (L.E.O. and M.D.B.). Any discrepancies were resolved through a consensus discussion between reviewers, and a senior author (D.L.B.) was consulted if a disagreement could not be resolved.

Studies were included if they: (1) included human participants, (2) participants who have sustained a primary ACL injury, (3) measured at least one biomarker of knee joint healing at more than one time point, and (4) published in either English or French. Grey literature including conference proceedings, letters to editors, case reports, clinical commentaries, pilot studies, and review articles were excluded.

The NIH Quality Assessment Tool for Before-After (Pre-Post) Studies With No Control Group was used to assess the quality of the final included papers *(*Supplemental Table 5.2.A*)*. Each study was independently assessed by two authors (L.E.O. and M.D.B.), and any disagreements were discussed until a consensus was reached. Articles were not excluded on the basis of the assessment.

A standardized data sheet was used to record the following information: sample size; participant age; time between injury/surgery and sample collection; biological sample(s) collected; biomarker(s) analyzed. One author (L.E.O.) recorded all pertinent data from the included articles, and another author (M.D.B.) independently reviewed these data for accuracy

and completeness. If the standard deviation was not reported, it was estimated according to a previously validated formula: (higher range value - lower range value)/4 or interquartile range/1.35 (Higgins et al., 2008). The primary outcome of interest was the biomarker reflecting knee joint healing in patients with ACL injuries according to i) biomarker(s) measured, and ii) biological tissue(s) collected for this analysis, and iii) the age of participants enrolled in the included studies. Additional outcomes of interest included the reported correlation coefficients between different biomarkers measured within studies, and the difference of biomarker levels between patients with ACL injuries and control participants, where applicable.

Results

Initial screening produced 857 studies, which included 372 duplicates, leaving a total of 485 studies to undergo title and abstract screening *(Figure 1)*. Of these, 152 studies were assessed for eligibility through full text screening and resulted in a total of six studies that were deemed eligible based on our pre-determined inclusion criteria and were then subject to extraction (Supplemental Table 5.1.A.) and quality assessment (Supplemental Table 5.2.A).

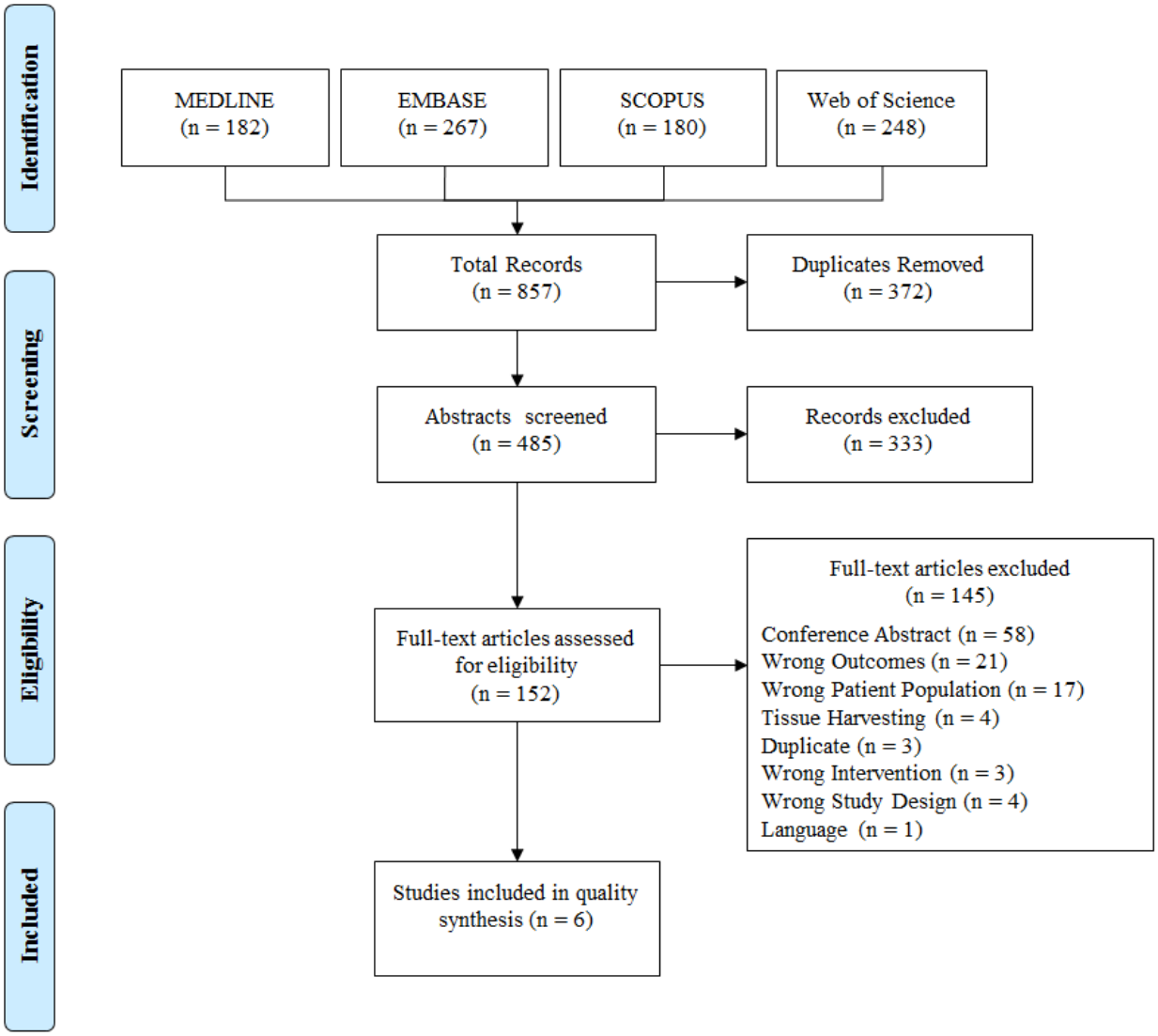

Figure 5.1. *Prisma flow diagram of study screening process according to inclusion criteria*

From the six included studies in this systematic review, the two most prevalent biomarkers were CTX-II and IL-6 (Table 5.2). These biomarkers reflected knee joint healing through consistent elevation and/or gradual decrease following an ACL reconstruction (Supplemental Table 5.1.A.). Anil et al. studied *sf*IL-6, a pro-inflammatory cytokine, in the early post-operative period following ACLR by measuring samples of synovial fluid prior to ACLR and at a mean time of 10 days following ACLR (range 4-32 days; Anil et al., 2019). There was a statistically significant increase in the concentration of *sf*IL-6 of 31.8 pg/mL before surgery to 4 023.2 pg/ml after surgery. Mendias et al. measured biomarkers of muscle atrophy, inflammation and cartilage turnover in patients undergoing ACLR and rehabilitation. Plasma samples for biomarker analysis (Supplemental Table 5.1.A.) were taken one week prior to ACLR, then 3 days, 2 weeks, 5 weeks, 12 weeks, 18 weeks and 26 weeks following ACLR (Mendias et al., 2013). The cartilage turnover biomarker COMP decreased immediately after surgery and returned to pre-op levels. In addition, the acute phase protein CRP was highly elevated immediately after surgery, and returned to pre-operative levels by second post-op visit (2 weeks post-op). Notably, levels of plasma IL-6 did not change significantly throughout the study. Larsson et al. (Larsson et al., 2017) studied pro-inflammatory cytokines (including *sf*IL-6) and cartilage metabolism (*u*CTX-II) biomarkers in patients undergoing ACLR. Participants were grouped into early ACLR, delayed ACLR, and rehabilitation alone. The lowest concentration of biomarkers was observed in the rehabilitation alone group and highest was in the early ACLR group. Early ACLR patients had statistically significantly higher cytokine concentrations at 4 months (*sf*IL-6, *sf*IL-8, *sf*IL-10, *sf*TNF), 8 months (*sf*IL-6, *sf*IL-8 and *sf*TNF) and at 5 years (*sf*IL-6). Both groups treated with ACLR had higher concentrations of sfIL-6 compared to the group treated with rehabilitation alone. *u*CTX-II continued to decrease at the 5-year mark, with a

plateau around 2/3 of the way through the 5-year study period. Chmielewski et al. (Chmielewski et al., 2012) studied *u*CTX-II, a biomarker of cartilage degradation, in patients with ACL injuries and uninjured controls at 4, 8, 12 and 16 weeks post ACLR. The control group concentration of uCTX-II across all time points was 2827.87 (5033.7) ng/mmol. In the ACLR group, the mean (SD) levels of *u*CTX-II were 3521.2 (4217.5), 3332.8 (3795.1), 3109.5 (3317.9), and 2850.7 (3185.5) ng/mmol at 4-, 8-, 12-, and 16-weeks post ACLR, respectively. These levels decreased across the time points but remained statistically significantly higher compared to the control baseline level. Tourville et al. (Tourville et al., 2013) studied the relationship between biomarkers of type II collagen metabolism (*u*C1, 2C – ratio of type I and type II cleavage product), *s*CPII (procollagen II C-propeptide), cleavage-to-synthesis ration [*u*CTX-II]/*s*CPII at baseline, 1 year and 4 years following ACLR. Patients with ACLR had an increased ratio of (*u*C1, 2C) to *s*CPII compared with controls at 1 and 4 year follow ups. They also had a significantly increased cleavage-to-synthesis of type II collagen ratio [*u*CTX-II]/*s*CPII compared with controls at 4-year follow up. Beynnon et al. studied biomarkers of cartilage metabolism in knee joint synovial fluid at 3, 4, 12, and 24 months following ACLR in both the injured and uninjured contralateral knee (Beynnon et al., 2005). These biomarkers were COL II ¾ Longmono (biomarker of cleavage of type II collagen), 864 (biomarker of turnover of aggrecan), and CPII (synthesis of type II collagen). They revealed that all three biomarkers were all elevated compared to the contralateral control knee and the control group at baseline. COL II ¾ Longmono returned to normal limits by the 12 month follow up interval. CPII remained elevated relative to normal at the 12- and 24-month intervals. 864 was elevated relative to normal at the 12-month time point.

Five studies evaluated adult populations (age range; Table 5.2). One study (average age 19.6±4.5) evaluated the effect of age on biomarker levels of knee joint healing and showed a negative correlation between age and CTX-II concentrations ($r=-.769$, $p < 0.001$) (Chmielewski et al., 2012).

Table 5.2

Biomarkers of knee joint healing after ACL reconstruction, and participant age, from included studies.

Study	**Anil, 2019**	**Beynnon, 2005**	**Chmielweski, 2012**	**Larsson, 2017**	**Mendias, 2013**	**Tourville, 2013**
Age (mean±SD)	33.4±10.7	32.7±6.5	19.6±4.5	26±4.9	28±2.4	28.9±10.25
Biomarker type *(biomarkers reoccurring in ≥3 studies)*	Cytokines, cartilage metabolism *(sfIL-6)*	Cartilage metabolism	Cartilage metabolism *(uCTX-II)*	Cytokines, cartilage/bone metabolism *(sfIL-6, uCTX-II)*	Muscle atrophy, cytokines, cartilage metabolism *(sIL-6)*	Cartilage metabolism *(uCTX-II)*

Abbrev: *CTX-II, C-terminal crosslinking telopeptide of type II collagen; IL-6, Interleukin-6; s, serum; sf, synovial fluid; u, urine.*

Discussion

The purpose of this review was to identify the most studied biomarkers assessing knee joint healing in patients with ACL injuries and identify the prevalence of adolescent participants in these studies. Six studies were retained which included a variety of biomarkers mainly of cartilage metabolism and inflammation *(Figure 1)*. CTX-II (Chmielewski et al., 2012; Larsson et al., 2017; Tourville et al., 2013) and IL-6 (Anil et al., 2019; Larsson et al., 2017; Mendias et al., 2013) were most commonly studied biomarkers and were included in three of the six studies. This aligned with our hypothesis that CTX-II, a commonly measured cartilage degradation biomarkers, and IL-6, a commonly measured biomarker of inflammation, would be the most prevalent. We also hypothesized that all study participants would be adults, which was the case for all but one study which did not exclude participants under the age of 18 (average age 19.6±4.5 (Chmielewski et al., 2012a)).

These findings are further supported by an unpublished scoping review which found that CTX-II was the most common biomarker for reflecting articular cartilage degradation as it related to knee OA severity. CTX-II can be measured in synovial fluid, but is more commonly measured in urine samples since they are less invasive to obtain (Chmielewski et al., 2012). The advantage of synovial fluid samples is the localization of CTX-II released in the knee joint, whereas urine will reflect CTX-II levels on a systemic level in the body. *u*CTX-II levels have been reported to increase with knee OA severity and be linked to knee OA progression (Garnero et al., 2001; Jordan et al., 2006; Tanishi et al., 2009). CTX-II is a by-product of articular cartilage degradation occurring when cartilage degradation exceeds the rate of cartilage synthesis in normal cartilage metabolism (Chmielewski et al., 2012). A cascade of pathogenic processes are initiated following an ACL injury, including that of increased turnover of type II collagen within days and/or weeks of the injury (Lohmander et al., 1999). There is also histological evidence of degeneration and a persistent increase in collagenase cleavage and denaturation of type II collagen within a year of ACL injury. This pathogenic presentation is significant as it is similar to that seen in idiopathic OA in the joint (Nelson et al., 2006).

Chmielewski et al., Larsson et al., and Tourville et al. are the three included studies in this review which looked at CTX-II as a biomarker of knee joint healing in patients with ACL injuries (Chmielewski et al., 2012; Larsson et al., 2012; Tourville et al., 2013). This biomarker was measured in either urine as outlined in Supplemental Table 5.1.A. Chmielewski et al. measured *u*CTX-II at 4-, 8-, 12- and 16- weeks following ACLR (Chmielewski et al., 2012). Given that the levels in the ACLR group were still elevated above that of uninjured controls and appeared to be continuing to approach baseline levels, additional time points could provide a clearer description of a potential plateau, increase, or decrease in *u*CTX-II in patients past 16

weeks following their ACLR. Additionally, an extended longitudinal timeline could capture levels of articular cartilage degradation as patients return to more regular activities and sports. 17-30% of adolescent athletes will sustain a second ACL injury within two years following an ACLR (Paterno et al., 2012, 2014; Shelbourne et al., 2009) and over 30% of these injuries will occur within the first 20 sport exposures (Paterno et al., 2014). Monitoring these levels could be equally as important and interesting around the time when patients are returning to activity given the increased risk for re-injury around this time.

Tourville et al. (Tourville et al., 2013) studied ratios of cartilage degradation (*u*CTX-II) to synthesis (*s*CPII), which provided a comprehensive and insightful reflection of cartilage metabolism in the knee joint, since degradation of articular cartilage exceeding the rate of synthesis increases is a hallmark of knee OA and a sign of increased risk (Bellido et al., 2011; Delmas, 2009). The participants who underwent ACLR were sub grouped into normal and abnormal joint space width narrowing (JSW). Increased and abnormal JSW is a characteristic of knee OA and is a measure of knee OA severity (Heidari, 2011). At the 4 year follow-up, the cleavage-to-synthesis ratio was significantly elevated in the abnormal group compared to the controls, but not for the normal JSW group compared to controls (Tourville et al., 2013). The significance of this study is the ability of the cleavage-synthesis ratio to distinguish between participants with a normal JSW difference and patients with an abnormal JSW difference. Abnormal JSW is indicative of early stages of knee OA and therefore poorer knee joint health (Chan et al., 2008). Future research studying cartilage metabolism ratios could provide impactful literature for clinicians and physiotherapists working with their patients recovering from ACLR.

Larsson et al. studied *u*CTX-II and *sf*IL-6 in patients with ACL injuries who underwent early ACLR, delayed ACLR or rehabilitation alone, over 5 years (Larsson et al., 2017). This

study draws attention to healing response over time with different treatment options. As previously reported, ACLR itself is an additional trauma to the knee joint (Jawa et al., 2011b). What this study highlights the influence had of time between injury and surgery on biomarkers of knee joint healing, as higher levels of biomarkers of degradation were reported longitudinally in patients who underwent early ACLR compared to a delayed ACLR, and compared to rehabilitation alone. This study also reported similar trends for *sf*IL-6 and *u*CTX-II reflecting knee joint healing over the 5 years of this longitudinal study. *sf*IL-6 decreased and began to plateau around the last year of the study, whereas *u*CTX-II was continuing to decrease even at the 5-year mark, with a plateau around 2/3 of the way through the 5-year study period. The prolonged changes of these biomarkers over 5 years post-operatively emphasizes the continuous process of knee joint healing as biomarkers slowly approach baseline levels years following the initial injury and surgery. This is remarkable as patients are often cleared for full return to activity between 6 and 9 months following their ACLR (McRae et al., 2011). This longitudinal study reveals that physiologically, the knee joint may still be in a vulnerable phase of healing during this RTA period which takes place so shortly following surgical reconstruction.

Cytokines are soluble factors produced by cells of the immune system in response to infection, inflammation, or trauma, and have been reported to increase cartilage degradation (Dinarello, 2000, 2007; Orita et al., 2011; Sorsa et al., 2004). Multiple inflammatory cytokine biomarkers, such as tumor necrosis factor-alpha, interleukin-1, and IL-6, are upregulated following a joint injury and have been shown to contribute to the cartilage degradation process (Cuellar et al., 2010; Goldring, 1999; Higuchi et al., 2006; Pola et al., 2005). Included studies in this review presented that cytokines are elevated immediately following injury and likely persist for a prolonged period (Anil et al., 2019; Larsson et al., 2017; Mendias et al., 2013). We

identified that along with CTX-II, IL-6 was equally as prevalent in existing literature reflecting knee joint healing following an ACL reconstruction, although local *sf*IL-6 was more significantly able to reflect knee joint healing compared to IL-6 measured in plasma. IL-6, which is considered a stress cytokine, acts locally and systemically to generate a variety of physiological responses (Jawa et al., 2011a). Levels of IL-6 are correlated with the extent of injury (Jawa et al., 2011a), as greater tissue trauma results in greater IL-6 release, so patients with more severe injuries exhibit elevated IL-6 levels.

Anil et al. reported an elevation of *sf*IL-6 in the immediate post-operative period following ACLR (Anil et al., 2019). As previously mentioned, Larsson et al. studied *sf*Il-6 along with *u*CTX-II in patients with ACL injuries who underwent early ACLR, delayed ACLR or rehabilitation alone, over 5 years (Larsson et al., 2017). They reported a steady decrease of *sf*IL-6 over 4 years, and a plateau during the fifth and final year of the study. IL-6 is thought to reduce expression of type II collagen and plays a role in the development of collagen induced arthritis (Anil et al., 2019). The presence of pro-inflammatory IL-6 in the knee acutely after ACLR might explain the lack of protection against the later development of knee OA (Anil et al., 2019). This elevation in the immediate post-operative period might explain the absence of the protective effect of ACLR in the development of OA in patients with ACL injuries.

Mendias et al. studied IL-6 as a biomarker of knee joint healing in patients following ACLR; however, they measured this biomarker in plasma as opposed to the local and gold standard of synovial fluid (Mendias et al., 2013). Surprisingly, the circulating plasma did not lead to the same increase in levels following injury and surgical reconstruction as previous literature where synovial fluid IL-6 was measured (Anil et al., 2019; Bigoni et al., 2013; Cameron et al., 1997; Cuellar et al., 2010; Hayward et al., 2011; Larsson et al., 2012). The

takeaway is that IL-6 should be measured in synovial fluid when investigating the reflection of this biomarker with respect to knee joint health in patients with ACL injuries.

The second outcome of interest from this review was the age of study participants. Only one study had an average age <20 years old (Table 5.2*;* Chmielewski et al., 2012). This study presented a correlation between the age of participants and biomarkers of healing (CTX-II; r=-.769, $p < 0.001$ (Chmielewski et al., 2012). The age of study participants is significant because adolescents experience physiological changes as they progress through puberty including growth plate activity and hormonal changes. Specifically, endochondral bone formation involves highly active chondrocytes and the secretion of type II collagen at the growth plates in the long bones of the leg. This secretion of type II collagen can affect levels of CTX-II in adolescent populations (Chmielewski et al., 2012), and underlines the possibility of active growth plates in adolescents interfering with the ability of CTX-II to reflect the healing process of the knee joint. Furthermore, the ability of cytokines to stimulate chondrocytes and cartilage degradation (Sorsa et al., 2004) adds to the uncertainty for how inflammatory biomarkers (e.g. IL-6) are impacted by the increase in growth plate activity and altered metabolic and endocrine environment present in adolescents. Considering these factors, it is illogical to extrapolate a physiological measure, like biomarkers of healing, from adults to adolescents. Thus, our systematic review emphasizes the importance of future research prioritizing adolescent populations.

ACL injuries are complex and can impact physiological, biomechanical and psychosocial components (Kvist et al., 2018). Therefore, research on ACL injuries used to guide rehabilitation should consider these three aspects. In this review we have focused on the physiological component, and the lack of research on adolescents. Further research with adolescent participants is necessary to build the scientific foundation of biomarkers of the healing process in the knee

joint. This should include multiple variables assessing inflammation (e.g. *sf*IL-6), cartilage health (e.g. *u*CTX-II), and synthesis (e.g. CPII). These physiological variables can be studied in combination with biomechanical variables, like limb symmetry indices and ligament laxity, which are the parameters currently in use when clearing patients for RTA (Noyes et al., 1991; Petersen et al., 2014; Petersen et al., 2013). Finally, collecting psychosocial variables like subjective pain, function and quality of life to would create more comprehensive and impactful research for clinicians and physiotherapists who develop and modify these RTA guidelines.

Based on the findings of our systematic review, we determined that there is very limited existing research and evidence on the use and findings with respect to biomarkers and healing status post ACL injury and surgical reconstruction. Our review found: *i)* only six studies evaluated physiological healing using biomarkers following an ACL reconstruction, *ii) u*CTX-II and *sf*IL-6 were the most studied biomarkers, and *iii)* there is a significant literature gap with respect to evaluating biomarkers of knee joint healing in adolescents following an ACL reconstruction. Adolescents are unique from adults due to growth (Kronenberg, 2003b) and sex hormone variation, indicating that biomarker research in adults should not be extrapolated to adolescents (Juul, 2001). Therefore, *i)* more research is needed specific to adolescents and, *ii)* this research should explore *u*CTX-II and *sf*IL-6 since they are the most studied biomarkers reflecting knee joint healing. Once research is conducted to exploring the correlation of IL-6 and CTX-II with knee joint health and healing in adolescents, we propose addressing all three components of ACL injuries including biomechanical, physiological and psychosocial. The inclusion of these data will provide clinicians and researchers alike a more holistic view on the healing status of the knee joint following an ACL reconstruction.

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Conflict of Interest

The authors have no professional relationships that stand to gain from the current study. The results of the present study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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APPENDIX

Supplemental Table 5.1.A
Characteristics of Included Studies

Reference	Sample Size	Age (years)	Time points measured	Sample	Biomarkers	Main Findings
Anil, 2019(Anil et al., 2019)	n=8	33.4±10.7	• Pre-op • Post-op 10.4 (4-32) days	• Synovial fluid	• *sf*MMP-3 • *sf*CCL5 • *sf*V-PLEX • *sf*bFGF • *sf*MSD • *sf*TIMP-1 • *sf*TIMP-2 • *sf*IL-1Ra • *sf*IL-6 • *sf*VEGF • *sf*MCP-1 • *sf*MIP-1b	• Statistically significant increase in concentration of IL-6 of 31.8 pg/mL before surgery to 4 023.2 pg/ml after surgery
Beynnon, 2005(Beynnon et al., 2005)	n=22	32.55 (18-44)	• Pre-op • Post-op 6 months • Post-op 12 moths • Post-op 24 months	• Synovial fluid	• *sf*CS846 • *sf*COL2-3/4C$_{longmonoiepitope}$ • *sf*CPII	• All biomarkers were elevated compared to control at baseline • COL II ¾ Longmono returned to baseline limits by 12 months post-op • CPII remained elevated relative to baseline at 12 and 24-months post-op • CS864 was elevated 12-months post-op
Chmielewski, 2012(Chmielewski et al., 2012)	n=28	19.6±4.5	• Post-op 4 weeks • Post-op 8 weeks • Post-op 12 weeks • Post-op 16 weeks	• Urine	• *u*CTX-II	• Control group levels of uCTX-II: 2827.87 (5033.7) ng/mmol • ACLR group levels of uCTX-II were 3521.2 (4217.5), 3332.8 (3795.1), 3109.5 (3317.9), and 2850.7 (3185.5) ng/mmol at 4, 8, 12, and 16 weeks post ACLR
Larsson, 2017(Larsson et al., 2017)	n=118	26±2.9	• Post-injury 0-6 weeks • Post-injury 4 months • Post-injury 8 months	• Synovial fluid • Serum	• *sf*IL-6 • *sf*IL-8 • *sf*IL-10	• Highest concentration in the early ACLR group

			• Post-injury 1 year • Post-injury 2 years	• Urine	• *sf*INFgamma • *sf*TNF		• Early ACLR higher concentrations at 4
Larsson, 2017(Larsson et al., 2017) *(cntd.)*			• Post-injury 5 years		• *sf*ARGS • *s*ARGS • *u*CTX-II • *u*NTX-I		months (*sf*IL-6, *sf*IL-8, *sf*IL-10, *sf*TNF), 8 months (sfIL-6, sfIL-8 and sfTNF) and at 5 years (*sf*IL-6). • ACLR had higher concentrations of *sf*IL-6 compared to the group treated with rehabilitation alone • *u*CTX-II plateau around 2-3 years, then continued to decrease after 5 years
Mendias, 2013(Mendias et al., 2013)	n=18	28±2.4	• Pre-op • Post-op 3 days • Post-op 2 weeks • Post-op 5 weeks • Post-op 12 weeks • Post-op 18 weeks • Post-op 26 weeks	• Plasma	• *p*CCL2 • *p*CCL3 • *p*CCL4 • *p*CCL5 • *p*EGF • *p*FGF-2 • *p*IL-1a • *p*IL-1b • *p*IL-6	• *p*IL-10 • *p*IL-ra • *p*TNF-a • *p*COMP • *p*CRP • *p*IGF-1 • *p*Myoglobin • *p*Myostatin • *p*TGF-b	• COMP decreased after surgery and returned to pre-op levels • CRP elevated immediately after surgery and returned to pre-op levels by second post-op visit (2 weeks post-op). • *p*IL-6 did not change significantly
Tourville, 2013(Tourville et al., 2013)	n=35	28.8	• Pre-op • Post-op 1 year • Post-op 4 years	• Serum • Urine	• *s*CPII • *u*C2C • *u*C1, 2C • *u*CTX-II		• Increased ratio of (uC1, 2C) to sCPII compared with controls at 1 and 4 years post-op • Significantly increased cleavage-to-synthesis of type II collagen ratio [uCTX-II]/sCPII compared with controls at 4 years post-op

Abbreviations *ARGS* ARGS neoepitope*, bFGF* Basic fibroblast growth factor*, C1* Type I Collagen, 2*C* Type II Collagen, *C2C* Cleavage of Type II Collagen, *CCL2* monocyte chemoattractant protein-1*, CCL3* monocyte chemoattractant protein-2, *CCL4* monocyte chemoattractant protein-3, *CCL5* monocyte chemoattractant protein-5*, COL2-3/4 Clongmono Epitope* Collagen Type II*, COMP* Cartilage Oligomeric Matrix Protein, *CPII* Synthesis of Type II Procollagen, *CRP* C-Reactive Protein, *CS846* Chondroitin Sulfate 846 Epitope, *CTX-II* C-Terminal Crosslinking Telopeptide of Type II Collagen, *EGF* Epidermal growth factor*, FGF-2* Fibroblast Growth Factor-2 *, IGF-1* Insulin-Like Growth Factor-1*, IL-1beta* Interleukin 1-beta, *IL-6* Interleukin-6, *IL-6Ra* Interleukin-6 Receptor

Alpha, *IL-8* Interleukin-8, *IL-10* Interleukin-10, *IFN-Gamma* Interferon-Gamma**,** *MCP-1* Monocyte chemoattractant protein-1, *MIP-1b* Macrophage Inflammatory Protein-1 beta*, MMP-3* Matrix Metaloproteinase-3, *NTX-I* N-Terminal Crosslinking Telopeptide of Type I Collagen, *p* Plasma, *s* Serum, *sf* Synovial Fluid, *TIMP-1* metallopeptidase inhibitor 1, *TIMP-2* metallopeptidase inhibitor *2, TGF*-beta Transforming Growth Factor beta, *TNF-alpha* Tumor Necrosis Factor-alpha, *u* Urine, *VEGF* Vascular endothelial growth factor.

Supplemental Table 5.2.A
NIH Quality Assessment of Included Studies

	Assessment Questions											
Reference	1	2	3	4	5	6	7	8	9	10	11	12
Anil, 2019(U. et al., 2019)	1	1	1	0*(NR)	0	1	1	0	0*(NR)	1	0	1
Beynnon, 2005(B.D. et al., 2005)	1	1	1	0	1	1	1	1	1	1	0	1
Chmielewski, 2012(Chmielewski et al., 2012)	1	1	1	1	1	1	1	0	1	1	0	1
Larsson, 2017(S. et al., 2017)	0	1	1	1	1	1	1	0	1	1	0	1
Mendias, 2013(Mendias et al., 2013)	1	1	1	1	0	1	1	0	0*(NR)	1	0	1
Tourville, 2013(Tourville et al., 2013)	1	1	1	1	1	0	1	0	1	1	0	1

Yes = 1; No = 0; *CD, cannot determine; NA, not applicable; NR, not reported

1. Was the study question or objective clearly stated?2. Were eligibility/selection criteria for the study population prespecified and clearly described?3. Were the participants in the study representative of those who would be eligible for the test/service/intervention in the general or clinical population of interest? 4. Were all eligible participants that met the prespecified entry criteria enrolled? 5. Was the sample size sufficiently large to provide confidence in the findings? 6. Was the test/service/intervention clearly described and delivered consistently across the study population? 7. Were the outcome measures prespecified, clearly defined, valid, reliable, and assessed consistently across all study participants? 8. Were the people assessing the outcomes blinded to the participants' exposures/interventions? 9. Was the loss to follow-up after baseline 20% or less? Were those lost to follow-up accounted for in the analysis? 10. Did the statistical methods examine changes in outcome measures from before to after the intervention? Were statistical tests done that provided p values for the pre-to-post changes? 11. Were outcome measures of interest taken multiple times before the intervention and multiple times after the intervention (i.e., did they use an interrupted time-series design) 12. If the intervention was conducted at a group level (e.g., a whole hospital, a community, etc.) did the statistical analysis take into account the use of individual-level data to determine effects at the group level?

CHAPTER 6: MANUSCRIPT 2

Biomarkers of Knee Joint Healing in Adolescents with Anterior Cruciate Ligament Injuries: Pilot Study

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Abstract

Objective: ACL injuries are increasing in adolescents and lead to an increased risk for early-onset knee osteoarthritis (OA). Interleukin-6 (IL-6), c-terminal crosslinking telopeptide of type II collagen (CTX-II) and procollagen type II collagen propeptide (PIICP) are biomarkers reflecting knee joint healing. There is currently no research on these biomarkers in adolescents who are unique due to physiological changes in puberty. The purpose of this study was to determine whether an association exists between urine (*u*) and synovial fluid (*sf*) biomarkers of healing, and the ratio of cartilage degradation to synthesis, in adolescents with ACL injuries.

Design: Urine and synovial fluid samples were collected prior to ACL reconstruction (ACLR). ELISA was used to measure levels of IL-6, CTX-II and PIICP. Multiple dilution factors were used to perform this analysis (*sf*CTX-II: 1:3, 1:10, 1:50; *sf*PIICP: 1:3, 1:7.5, 1:15; IL-6: 1:3), where PIICP 1:15 and CTX-II 1:50 was used for analysis. Spearman's Rho (r_s) correlation coefficients were calculated to determine the association between *u*CTX-II/*sf*CTX-II, *u*IL-6/*sf*IL-6. A ratio of PIICP: CTX-II was calculated.

Results: Due to undetectable biomarker levels in multiple samples, we only report r_s for *u*CTX-II/*sf*CTX-II (r_s = -.200, *p*-value = .800, n=4). We also reported a ratio for *sf*PIICP: *sf*CTX-II (1:50; 23.06 ±19.23).

Conclusions: This is the first study to explore IL-6, CTX-II, and PIICP in adolescents with ACL injuries. Our findings indicate that sample collection and preparation require further research in order to reliably interpret these results. This includes testing synovial fluid at multiple dilution factors and enzymatic digestion. Future research building on this pilot data can provide a better understanding of biomarker analysis. The feasibility of these biomarkers reflecting joint healing longitudinally can then be studied in adolescents recovering from ACL reconstruction.

Key Words: Adolescents, Anterior Cruciate Ligament (ACL), Articular Cartilage Degradation, C-Terminal Crosslinking Telopeptide of Type II Collagen (CTX-II), Interleukin-6 (IL-6), *PIICP* Procollagen Type II Collagen Propeptide, *n* sample size, sf synovial fluid, *u* urine

Introduction

Anterior cruciate ligament (ACL) injuries have increased 2.3% annually in adolescents between 1993-2013 (Beck et al., 2017) and have been associated with an increased risk for early-onset knee osteoarthritis (OA; Guilak, 2011). Up to 62-80% of patients show radiographic signs of knee OA within 10-15 years following their initial ACL injury (Øiestad et al., 2010). The progression to OA following an ACL injury is multifactorial (Friel & Chu, 2013), but is often due to abnormal mechanical loading patterns causing an increase in cartilage degradation and inflammation in the joint (Andriacchi et al., 2004; Felson et al., 2000; Goldring, 2000, 2012; Noyes, 2009). Consequences of late-stage knee OA include structural joint damage, functional limitations and persistent pain which negatively impacts a patient's quality of life (Stefan et al., 2007).

ACL injuries are multifaceted in nature, with biomechanical, physiological and psychosocial components (Kvist et al., 2018). One clear limitation of current rehabilitation guidelines is the failure to consider a physiological reflection of healing in the knee joint. As the incidence of knee OA following ACL reconstruction remains as high as 87% (Nakata et al., 2008; Shelbourne et al., 1997), biomarkers of healing could provide insight regarding when it is most appropriate to introduce an increased mechanical loading to the healing knee joint.

Biomarkers are used as objective measures of cellular biochemical function, and can be analyzed in serum or synovial fluid, and through non-invasive biological samples like urine and saliva (Biomarkers Definitions Working Group., 2001; Strimbu et al., 2010). Analyses of biomarkers allows assessments of physiological properties of the knee joint's healing process by measuring, for example, the by-products of the metabolic processes of bone and cartilage which is a potential indicator of knee OA risk (Garnero et al., 2001; Nguyen et al., 2017). Our recent

systematic review concluded that the two most studied biomarkers reflecting knee joint healing in patients with ACL injuries were c-terminal cross-linking telopeptide of type II collagen (CTX-II; biomarker of cartilage degradation) and interleukin-6 (IL-6; pro-inflammatory biomarker; Ek Orloff et al., *under review*). In addition to these two biomarkers independently reflecting knee joint health in adults, procollagen type II collagen propeptide (PIICP) is a biomarker of cartilage synthesis, and as a ratio along with CTX-II has also been reported to reflect healing (Beynnon et al., 2005). The ratio of PIICP: CTX-II provides a more comprehensive reflection of degradation relative to the synthesis working to restore the type II collagen that is lost. These biomarkers can be locally measured in synovial fluid (*sf*), but are more commonly measured in urine samples which are simpler and less invasive to obtain (Chmielewski et al., 2012). The advantage of *sf* samples is the localization of CTX-II, PIICP and IL-6 released in the knee joint, whereas urine will reflect levels on a systemic scale in the body. The challenge of local synovial fluid sampling is the invasive nature of this procedure.

Endochondral bone development is the process through which all bones grow to generate the skeleton (Kronenberg, 2003). The epiphyseal plate, more commonly referred to as the growth plate, is a cartilaginous structure which drives the growth of long bones in length and is responsible for growth in height, especially notable during adolescence (Lui et al., 2014; Shim, 2015; Buckwalter et al. 1998; Fox et al. 2009). It is a non-vascularized hyaline cartilage and specialized connective tissue composed of two main components: chondrocyte cells and extracellular matrix (ECM) made up of water, proteoglycans (Hussain et al., 2016), and type II collagen providing tensile strength (Buckwalter et al., 1998; Fox et al., 2009). Chondrocytes are highly specialized metabolically active cells involved in the synthesis, degradation, maintenance and repair of the cartilage's ECM using degradative enzymes and are sparsely distributed

throughout the ECM (Archer et al., 2003; Buckwalter et al., 1998; Fox et al., 2009). These cells function through chondrogenesis and are vital for proper articular cartilage maintenance (Goldring, 2012). The chondrogenesis and cartilage metabolism that occurs in the growth plate is identical to what occurs to maintain the articular cartilage structure, primarily impacted by knee OA during normal metabolic maintenance. Given that growth plates are uniquely present in the pediatric and adolescent populations, and disappear through fusion following adolescence (Kronenberg, 2003), this additional source of cartilage metabolism in growing adolescents provides motivation to study these biomarkers of knee joint healing in this growing population. In addition, Chmielewski et al. reported increased levels of *u*CTX-II in participants under the age of 18 compared to their adult participants when studying this biomarker in patients following ACL reconstruction over 16 weeks following their reconstruction surgery (Chmielewski. et al., 2012).

Cytokine's ability to stimulate chondrocytes and cartilage degradation (Sorsa et al., 2004) adds to the uncertainty of how IL-6 and other inflammatory biomarkers are impacted by the increase in growth plate activity and altered metabolic and endocrine environment uniquely present in adolescents. In fact, as revealed through our review (Ek Orloff*, under review*), and supported by a previous review (Harkey et al., 2015) these measures are yet to be reported in a population made up solely of adolescents undergoing puberty. Thus, analyzing associations between local (*sf*) and systemic (*u*) levels of biomarkers of knee joint healing in adolescents with ACL injuries could provide new findings regarding systemic and non-invasive biomarkers able to reflect knee joint healing, and provide basis for future research and implementation by clinicians. The purpose of this study was to determine whether an association exists between local and systemic levels of CTX-II, IL-6, and PCIIP: CTX-II in adolescents with ACL injuries.

Methods

Participants

Ten patients with ACL injuries were enrolled from CHEO. Participants were required to have had no history of *i)* an arthritic condition (i.e. osteoarthrosis, rheumatoid arthritis, juvenile idiopathic arthritis), *ii)* previous traumatic lower-limb or knee injury (i.e. meniscal tear, ligament rupture, broken bones), *iii)* any inflammatory disease or immunocompromised (Lattermann. et al., 2016), or *iv)* prolonged steroid use.

Protocol

Consent Form and Questionnaires

Prior to data collections all participants read and provided verbal consent based on a consent form approved by the CHEO Research Institute Ethics Board and the University of Ottawa Research Ethics Board. Participants completed a participant information questionnaire (age, sex, injury date, surgery date, height, weight), and a pubescent-stage self-assessment form (Tanner; Taylor et al., 2001). Participants completed these forms virtually through a research survey distribution platform called RedCap.

Biological Sample Collection and Storage

Participants were asked to refrain from any high-impact activity in the 24-hours prior to biological sample collection to avoid influencing CTX-II levels (O'Kane et al., 2006). Participants were mailed a sterile specimen container, ice pack, and instruction sheet on how to collect, store and transport their own urine sample. Urine samples were collected the day before surgery (Chmielewski et al., 2012; Dam et al., 2009) between 12:00pm and 4:00pm (Gordon et al., 2008; Kong et al., 2006). Participants arrived for their scheduled ACL reconstruction surgery where synovial fluid samples were collected through aspiration to dryness with a syringe without

saline flushing at the time of their surgery performed by orthopedic pediatric surgeons (SC, KK). Biological samples were temporarily stored on ice and then centrifuged at 3000 rpm for 20 minutes and the supernatant was aliquoted and stored in duplicates at -80 °C prior to analysis.

Sample Analysis

Urine and synovial fluid samples were retrieved and measured at 450 nm using the Synergy HT Spectrophotometer Plate Reader (BioTek Instruments Inc., Winooski, VT, USA). Biomarker concentrations were measured using ELISA kits for levels of CTX-II (MyBioSource Inc, San Diego, California, USA), PIICP (MyBioSource Inc, San Diego, California, USA), IL-6 (Abcam Inc, Toronto, ON). Urine samples were analyzed for levels of creatinine, and urinary biomarkers of CTX-II, PIICP, and IL-6 were corrected for creatinine using the formula:

$$\text{Corrected CTX-II value (ng/mmol)} = 1000 \times \frac{\text{Urine CTX-II (ug/L)}}{\text{Creatinine (mmol/L)}} \quad (1)$$

(Cayman Chemical Company, Ann Arbor, MI, USA; Chmielewski et al., 2012). The concentration of creatinine (mmol/L) was determined using Enzyme Linked Immunosorbent Assay (Cayman Chemical Company, Ann Arbor, MI, USA).

Table 6.1
Coefficient of variation in percentage for each biomarker concentration measured in urine and synovial fluid

Biomarker	Mean coefficient of variation (%)	Standard deviation
*u*IL6	47.55	49.14
*sf*IL6	50.43	46.74
*u*CTXii	-	-
*sf*CTX-II (1:3)	10.04	9.66
*sf*CTX-II (1:10)	6.46	8.24
*sf*CTX-II (1:50)	13.79	15.18
*u*PIICP	33	32.40
*sf*PIICP (1:15)	1.90	2.05

Note. – indicates no data available
Abbrv. CTX-II C-Terminal Cross Linking Telopeptide of Type II Collagen, *IL-6* Inrerleukin-6, *PIICP* Procollagen Type II Collagen Propeptide, *n* sample size, sf synovial fluid, *u* urine.

Statistical Analysis

Two variables; the concentrations of the local synovial fluid biomarker (*sf*CTX-II, or *sf*IL-6) and its matched systemic urinary biomarker (*u*CTX-II, or *u*IL-6), were used in this analysis. The null hypothesis was that there is no correlation between the concentrations of the local and systemic level of each biomarker in adolescents with ACL injuries. Given the data violated the assumption of normality, Spearman's rho correlation coefficients were used to determine the concentration of the local synovial fluid biomarker in pg/mL and the creatinine-corrected concentration of systemic urinary in mmol/mL (Chmielewski et al., 2012). The Spearman's rho correlation coefficients were then extracted to determine the strength of association of the coefficient value with a strong correlation >0.7, moderate, 0.4-0.7, and low <0.4 (Field, 2015). A significance level of $p<0.05$ was used for all statistical tests and were performed using SPSS software version 17.0 (IBM, Chicago, IL, USA).

Results

Seven of the 10 adolescent patients with ACL injuries enrolled in this study provided urine samples, synovial fluid samples and online questionnaires. Partial data sets are available for the remaining three participants due to inability to provide one of the biological samples (Table 6.2.). Results of participant characteristics and biomarker concentrations can be found in Table 6.2. It is important to note that some samples resulted in undetected biomarker levels as outlined in Table 6.2.

Table 6.2

Descriptive statistics of participant demographics and biomarkers measured in urine and synovial fluid in adolescents with anterior cruciate ligament (ACL) injuries. Urine samples were collected the day prior to ACL reconstruction surgery, synovial fluid samples were collected directly prior to ACL reconstruction

Variable	n	Minimum	Maximum	Mean	Standard Deviation
Sex	*Male* (n=4), *Female* (n=6)	-	-	-	-
Age	10	12	17	14.40	1.65
Tanner Stage	7	3	5	4.2857	.76
BMI (kg/m^2)	9	15.98	30.40	23.8189	4.58
Injury to Surgery (days)	7	66.0	746.99	199.7143	244.54
*u*IL6 (ng/mmol)	4	.08	1.02	.4494	.45
*u*CTX-II (ng/mmol)	4	1.12	5.52	2.6310	1.98
*sf*CTX-II 1:3 (pg/mL)	6	9.80	1085.10	537.4333	505.26
*sf*CTX-II 1:10 (pg/mL)	8	69.63	5604.06	1660.63	2095.42
*sf*CTX-II 1:50 (pg/mL)	8	420.20	10127.05	2787.55	3423.16
*u*PIICP (ng/mmol)	4	.11	1.2975	1.2975	.84
*sf*PIICP 1:3 (pg/mL)	8	*ad*	*ad*	*ad*	*ad*
*sf*PIICP 1:7.5 (ng/mL)	8	1482.30	5707.10	29118.79	1084.39
*sf*PIICP 1:15 (pg/mL)	8	16186.07	32946.09	22663.30	6581.50

Note. – indicates no data available.

Abbrv. ad above detection range, *CTX-II* C-Terminal Cross Linking Telopeptide of Type II Collagen, *IL-6* Interleukin-6, *PIICP* Procollagen Type II Collagen Propeptide, *n* sample size, sf synovial fluid, *u* urine.

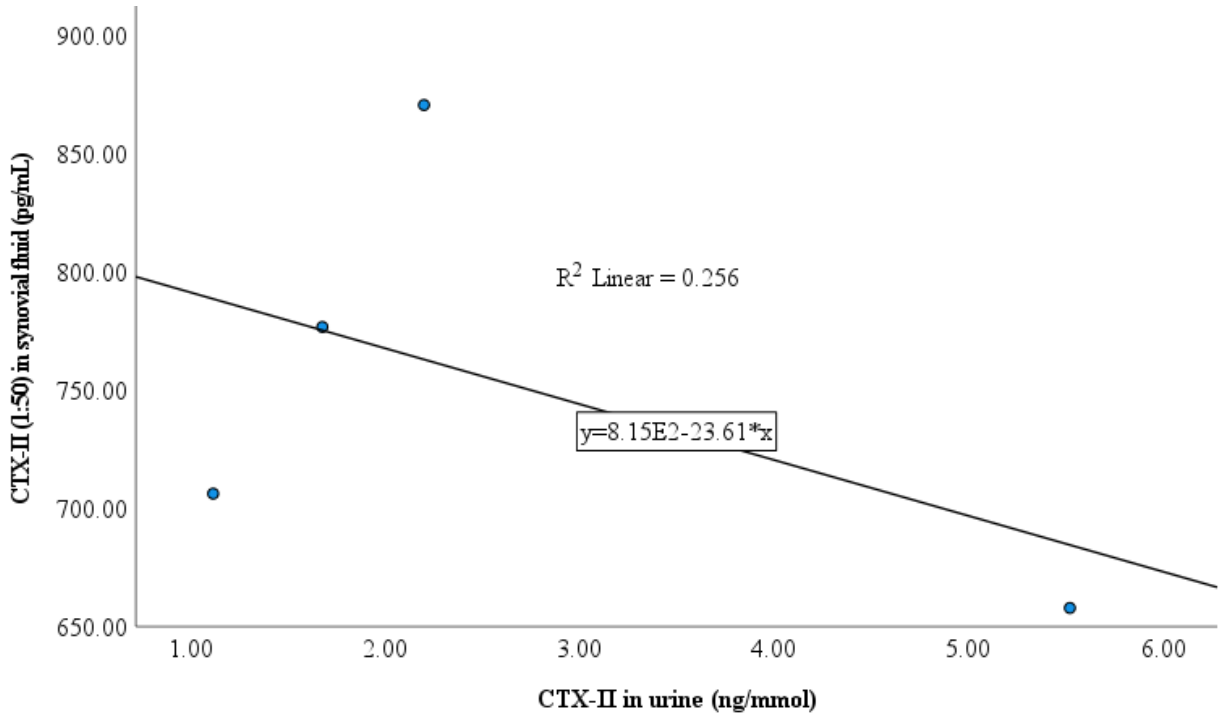

Figure 6.1. Spearman's Rho correlation coefficient between biomarkers of cartilage degredation (C-terminal cross-linking telopeptide of type II collagen (CTX-II) in urine (*u*) and synovial fluid (*sf*) in adolescentes with anterior cruciate ligament (ACL) injuries (r_s = -.200, *p*-value = .800, n=4).

Local and Systemic Biomarkers

CTX-II assay was performed three times at three separate dilutions (1:3, 1:10, and 1:50) where 1:50 was used for statistical analysis. Biomarker concentrations from IL-6, CTX-II, and PIICP in urine and synovial fluid can be found in Table 6.2. The concentrations of several urine samples were under the detection range of the assays. The correlation between local and systemic levels of CTX-II was r_s = -.200, *p*-value = .800, n=4.

Ratio of Cartilage Synthesis to Degradation

The ratio calculated of PIICP: CTX-II is available in synovial fluid samples at three separate dilution factors for CTX-II (Table 6.4). This ratio at 1:3 dilution of CTX-II was 348.88 +/- 624.13, for dilution of 1:10 was 91.74 +/- 117.85, and for a dilution of 1:50 was 23.06 +/- 19.23).

Table 6.3
Spearman's rho correlation coefficients between synovial fluid and urine levels of biomarkers of inflammation and cartilage metabolism

Correlation Variables	Correlation Coefficient	*p*-value	Sample Size (n)
*u*CTX-II/*sf*CTX-II	-.200	.800	4
*u*IL6/*sf*IL6	-	-	2

Note. – indicates no data available
Abbrv. CTX-II C-Terminal Cross Linking Telopeptide of Type II Collagen, *IL-6* Inrerleukin-6, *PIICP* Procollagen Type II Collagen Propeptide, *n* sample size, sf synovial fluid, *u* urine.

Table 6.4
Ratio of PIICP and CTX-II at various dilutions for ELISA analysis in synovial fluid of adolescents with ACL injuries

Participant ID	N	Mean	Standard Deviation	Minimum	Maximum
*sf*PIICP (1:7.5) / *sf*CTX-II (1:3)	6	45.52	82.61	1.66	212.68
*sf*PIICP (1:7.5) / *sf*CTX-II (1:10)	8	11.62	14.12	.62	36.97
*sf*PIICP (1:7.5) / *sf*CTX-II (1:50)	8	2.98	2.36	.44	6.13
sfPIICP (1:15) /*sf*CTX-II (1:3)	6	348.88	642.13	17.06	1648.28
sfPIICP (1:15) /*sf*CTX-II (1:10)	8	91.74	117.85	5.40	332.46
*sf*PIICP (1:15) /*sf*CTX-II (1:50)	8	23.06	19.23	3.44	55.06

Note. – indicates no data available
Abbrv. CTX-II C-Terminal Cross Linking Telopeptide of Type II Collagen, *IL-6* Inrerleukin-6, *PIICP* Procollagen Type II Collagen Propeptide, *n* sample size, sf synovial fluid, *u* urine.

Discussion

The purpose of this study was to be the first to examine the feasibility of measuring and examining the presence of associations between local synovial fluid, and systemic urine samples of biomarkers of knee joint healing. We posed the research questions: what is the association between concentrations of a) *sf*CTX-II and *u*CTX-II, b) *sf*IL-6 and *u*IL-6, and c) sfPIICP: *sf*CTX-II and uPIICP: uCTX-II, in adolescent patients with ACL injuries? Full data sets were collected for 7/10 recruited participants which entailed the collection of a urine and synovial fluid sample (Table 6.2.). Following analysis, the concentration in certain samples was outside the detection range as indicated in the sample size (*n*) column in Table 6.2. Synovial fluid CTX-II and PIICP were also assayed with various dilution factors in order to determine the optimal sample preparation for the assay (Table 6.2.).

Urine and Synovial Fluid Biomarkers

Due to the number of undetectable biomarkers in certain samples of *u*IL6 and *u*PIICP, a Spearman's Rho (r_s) correlation coefficient was computed to address the research question of the association between biomarker levels in local synovial fluid and systemic urine only for CTX-II (r_s = -.200, *p*-value = .800, n=4). The hypothesis for this research question where *u*CTX-II was moderately correlated (r=.309-.622) with knee OA severity (Garnero et al., 2001; Jordan et al., 2006; Tanishi et al., 2009), therefore reflecting the health of the internal knee joint. This weak correlation coefficient is not aligning with the hypothesized results. Referring to Table 6.1, we can extract the mean coefficient of variation for each assay where sample analysis was performed in duplicates. There was no available CV% for *u*CTX-II due to a lack of detectable sample analysis from the duplicates performed, and the CV% for *sf*CTX-II was 10.04, 6.46, and 13.79 for a dilution factor of 1:3, 1:10, and 1:50, respectively. When measured in duplicates, in

order to achieve an acceptable assay sensitivity, the intra-assay CV should be less than 10% (Conrad, 2018). Most dilution factors for CTX-II approximate this value, with the dilution factor of 1:10 providing the lowest and most reliable CV at 6.46%. Given the lack of a CV for *u*CTX-II, it is difficult to conclude the reliability of the association between local and systemic CTX-II.

Despite the few data points available to examine associations between local and systemic biomarkers, we were still able to compare the preliminary results to concentrations presented in both ACL injured and uninjured participants in existing literature. Previous research has reported increased concentrations of cartilage degradation and synthesis in urine and synovial fluid in adults with ACL injuries compared to uninjured controls (Chmielewski et al., 2012; Lohmander et al., 2003). In addition, increased levels have been reported in adolescents compared to adults likely due to increased growth plate activity chondrogenesis necessary for bones as they grow and fuse once finished growing in length (Chmielewski et al., 2012; Kronenberg, 2003). As reported by Chmielewski et al., patients with ACL injuries had *u*CTX-II concentrations of 3521.2 ± 4217.5, 3332.8 ± 3795.10, 3190.50 ± 3317.90, and 2850.70 ±3185.50 ng/mmol at 4-, 8-, 12- and 16-weeks post-operative, respectively. In addition, the mean *u*CTX-II concentration across testing time points was 2827.8 ± 5033.7 ng/mmol for uninjured controls (Chmielewski et al., 2012), with an intra-assay CV of 8.5% ± 8.9%. Our mean concentration of *u*CTX-II was 2.6310 ± 1.98 ng/mmol. These values are significantly lower compared to what we would expect given they are lower than values in patients with ACL injuries, and uninjured controls in mostly adult participants. It is also important to note that of the nine urine samples tested five had levels lower than the detection range (Table 6.2.). In order to improve upon the reliability of the results, aspects of sample collection and analysis could be addressed. Samples could be collected earlier in the morning with first or second void urine in order to increase the concentration of the urine

which may lead to biomarkers in the detectable range of the current assay. To improve upon sensitivity of the assay, samples could be analyzed in triplicates as opposed to duplicates. In addition, future analyses could evaluate samples using a western blot given the increased sensitivity of this method (Karlsson et al., 1989). Diluting samples from a range of 1:2 to 1:60 as performed by Chmielewski et al. should also be explored given their methodology yielded high and detectable concentrations of this biomarker in uninjured controls. Collecting and analysing plasma levels as an additional systemic biomarker could also prove relevant to confirm the level of this biomarker in the systemic urine samples.

Similarly, for *u*IL-6 and *u*PIICP (Table 6.2.) 5/9 urine samples came up under the detection range. For the four detectable samples, we reported a mean *u*IL-6 concentration of .45 ± .45 ng/mmol. These low levels ranging from undetectable to .45 ± .45 ng/mmol are more similar to the values reported in asymptomatic pediatric and adolescent participants recovered from urinary tract infections (Rodríguez et al., 2008). These samples were only in the detectable range for patients they were fighting a bacterial infection. This is not consistent with Park et al. who reported *u*IL-6 levels in arthritic adult patients were all in the detectable range (Park et al., 2016). It is possible that there is not enough circulating IL-6 in this adolescent population with ACL injuries population to allow for ELISA as an effective biomarker under these circumstances as cytokine levels increase with age and chronic disease (Rea et al., 2018). Nonetheless, it would be interesting to repeat this analysis while patients are recovering from their ACLR which acts as a secondary trauma leading to an increase in this acute phase cytokine (Jawa et al., 2011).

Anil et al. reported IL-6 synovial fluid levels of 31.80 pg/mL in adult patients prior to their ACL reconstruction surgeries, and 4 023.20 pg/ml following reconstruction. This compares to the reported concentrations from this study in our adolescent population prior to ACLR of

83.00 ± 143.45 pg/mL. This value is slightly increased compared to adults pre-operatively, which is in accordance with what we would expect in this younger population. Literature reports that IL-6 levels are upregulated following a joint injury and contribute to cartilage degradation (Higuchi et al., 2006; Pola et al., 2005). IL-6 has also been identified as an inhibitor of chondrogenesis (Friel et al., 2013). Given the increased activity of chondrogenesis in adolescents as cartilage and bone remodelling occur to grow and fuse long bones, an increased level of IL-6 would be expected in this adolescent population proportional to the increase in chondrogenesis.

Unlike the urine samples, most synovial fluid samples were able to produce a value in the detectable range, except two samples analyzed for 1:3 dilution of *sf*CTX-II. Analysis for PIICP concentrations in synovial fluid was performed twice. The first analysis was performed with samples using a dilution factor of 3. This produced values above the detection range, so upon consulting previous work (Nelson et al., 1998; Shinmei et al., 1993) this analysis was repeated with a dilution factor of 7.5 and then 15. The increased dilution resulted in a stronger signal during analysis, and the values obtained when the dilution factor was 15 are presented in Table 6.2. Analysis for CTX-II was performed using three dilution factors (1:3, 1:10, and 1:50; Table 6.2.), where the increase in dilution increased the signal of the assay and resulted in higher concentration readings. The dilution factor for the values presented for IL-6 in synovial fluid was 3.

Our reported concentrations of PIICP in synovial fluid was 22663.30 ± 66 (pg/mL), with a CV of 1.90 ± 2.05%, which is higher than what was reported in adult patients with OA (median 8800 pg/mL), and RA (median 8350 pg/mL; Nelson et al., 1998). Since adolescents experience increased collagen synthesis during bone growth in length, as cartilage is synthesized to then be replaced by bone until mature bone length is achieved (Kronenberg, 2003a). These results are

therefore in line with what we would expect to see in this population compared to literature in adults. Chmielewski et al also reported increased levels of cartilage degradation (*u*CTX-II) in younger adolescents following ACL injuries (Chmielewski et al., 2012), so an increased synthesis is necessary to counteract for the increased degradation.

As previously mentioned, we were expecting increased levels of urine and synovial fluid CTX-II in adolescents. Given the multiple undetected values in *u*CTX-II and the low values of *sf*CTX-II, this does not align with our hypothesis and with the literature. Lohmander et al. reported a mean *sf*CTX-II concentration of 9500 (1000-63200) pg/mL in adults with ACL injuries, which is much higher than our mean reported value of 537.4333 ± 505.26 pg/mL, 1660.63 ± 2095.42, 2787.55 ± 3423.16 at 1:30, 1:10, and 1:50 dilution factors, respectively. One speculation could have been that the low levels of degradation were attributed to the lack of activity and load bearing on the injured joint in the months between injury and surgery. The mean time between injury and surgery was 199.71 ± 244.54 days (Table 6.3), although with one outlier removed of 746 days, the mean is 108.6667 ± 46.12 days. The majority of participants experienced around 3.5 months following injury where they had a decrease in load bearing in their knee, which is similar to what was reported in literature by Beynnon et al., where mean time between injury and surgery was 90.7 (36-260) days in an accelerated group, and 125 (48-338) days in a non-accelerated group (Beynnon. et al., 2005) where cartilage synthesis and degradation were increased following ACL injury. However, Price et al. reported that type II collagen degradation occurs early following ACL injury and is not likely to be a direct result of mechanical loading since this was also observed in low-weight bearing sites of articular cartilage in the knee (Price et al., 1999). A more likely explanation could be the preparation of the synovial fluid prior to analysis.

Synovial fluid analysis is challenging due to its complex matrix and viscous non-homogenous matrix (Fam et al., 2007). Methods to reduce synovial viscosity like dilution and/or hyaluronidase (HAse) treatment are routinely used before a variety of analytic techniques (Song et al., 2012; Mateos et al., 2012; Sugiuchi et al., 2005; de Jager et al., 2007; Ekmann et al., 2010). As we observed when increasing the dilution factor for the second analyses of *sf*PIICP and *sf*CTX-II,increased dilution in addition to HAse treatment should be performed when repeating this analysis to ensure the synovial fluid viscosity is most accommodating for the binding of antigen to antibody required to obtain results when using ELISA. We can therefore assume that a dilution factor of 3 was suboptimal and resulted in lower levels of sfCTX-II than expected. These updated results would also aid in understand the low and undetected levels of *u*CTX-II given that the systemic level should reflect the local environment. The *u*CTX-II levels are therefore difficult to interpret prior to revision of the *sf*CTX-II analysis.

As discussed previously, when bones grow in length prior to adulthood, cartilage synthesis is replaced by bone through degradation of cartilage and synthesis of new bone. This leads to an increase in cartilage metabolism in adolescents (Kronenberg, 2003). In young children and adolescents, serum procollagen type II collagen propeptide is elevated compared to adults (Carey et al, 1997; Boeth et al, 2017). In this study, we reported a ratio of PIICP to CTX-II in synovial fluid was 348.88 ±642.13, 91.74 ±117.85, and 23.07 ± 19.23 for dilutions of CTX-II of 1:3, 1:10, and 1:50 respectively. As dilution of synovial fluid increases, the ratio decreases. A ratio where synthesis is proportionally increased compared to degradation is physiologically sound in a population without an arthritic condition. As briefly discussed, further analysis on these samples under various conditions of dilution and HAse digestion should be performed in order to determine the optimized preparation of these samples in order to better interpret the

results with more reliability. What these preliminary results may suggest, is that despite the unexpected values, these ratios may still be reflecting the metabolic activity proportionally but not providing strong enough standalone concentration values at this time.

Limitations

The limitations in the current study are notably the small sample size, and the number of undetected biomarker levels. This is a pilot study with the aim of being the first study to explore local and systemic biomarkers of knee joint healing in adolescents with ACL injuries. The preliminary and exploratory nature emphasized throughout this study focuses on the need for methodological improvement in further analysis. A small sample size is ideal to begin this exploratory process, as we can use the results from this study to improve upon the outcomes in a full data set with a sample size of 19 or higher, as deemed the necessary sample size based on an a priori power analysis.

While some biomarkers were undetected in samples, and multiple dilution factors were performed, these are necessary steps in order to produce reliable results in a future full study. Due to the limitation that we can only present partial results in this study, it is difficult to fully addressing the research questions. None the less, we are able to build upon these results and present a more complete analysis and interpretation once further research is conducted.

Conclusion

The initial aim of this pilot study was to assess the feasibility of measuring the biomarkers of knee joint healing IL-6, CTX-II, and PIICP in adolescents with ACL injuries. Our main findings are that an addition research step is required to determine the optimal methods and sample preparation to produce reliable results prior to proceeding with this feasibility study.

Biomarkers of knee joint healing have the potential to add an insightful perspective on the physiological healing process of the knee joint following ACL injuries in adolescents excited about their athletic careers and overall wellbeing. Reducing the risk for re-injury and early on set knee OA through increased monitoring of the recovery process following ACLR could result in healthier and more active future generations. This study with preliminary results is a small, but a crucial first step towards determining which biomarkers to measure, and how to interpret them in adolescent patients with ACL injuries. Measuring these biomarkers over the course of a patient's recovery from ACL reconstruction and throughout rehabilitation is a necessary future direction to track trends and changes over time prior to their return to sport and unrestricted activity. An increased sample size, longitudinal study design, control group and optimized sample preparation are all important aspects to include in this future research. This could bring us closer to the end goal of providing clinicians and physiotherapists with scientific and evidence-based tools they that can be implemented in order to improve guidelines for return to activity following ACL reconstruction surgery.

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Conflict of Interest

The authors have no professional relationships that stand to gain from the current study. The results of the present study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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APPENDIX

Supplemental Table 6.1.A
Participant characteristics and patient reported outcome measures.

Participant ID	*Age*	*BMI (kg/m^2)*	*Tanner Stage*	*Sex*
09	13	27.3	5	Female
05	14	22.3	4	Female
03	13	28.13	3	Female
06	17	21.13	4	Male
08	14	15.98	5	Female
10	15	24	5	Female
11	12	25.7	4	Female
01	15	30.4	-	Male
02	14	19.43	-	Male
04	17	-	-	Male

Note. – indicates no data available
Abbrv. BMI Body Mass Index.

Supplemental Table 6.2.A

Concentrations of all biomarkers of cartilage metabolism and inflammation measured in synovial fluid and urine, where urine concentrations have been corrected for creatinine (1).

Participant ID	uCreatinine (mg/dL)	*u*CTXii (ng/mmol)	*sf*CTXii (1:3) (pg/mL)	*sf*CTXii (1:10) (pg/mL)	*sf*CTXii (1:50) (pg/mL)	*u*IL6 (ng/mmol)	*sf*IL6 (pg/mL)	*u*PIICP (ng/mmol)	*u*PIICP (1:7.5) (ng/mL)	*sf*PIICP (1:15) (pg/mL)
AB09	116.98	*ud*	1085.07	5604.06	10127.05	.08	37.16	*ud*	10127.05	34809.84
AB05	273.41	1.12	9.82	201.93	706.10	.10	2.40	*ud*	706.1	16186.07
AB03	137.23	1.68	116.18	425.32	776.50	*ud*	162.68	*ud*	776.5	26525.41
AA06	250.36	5.52	*ud*	131.79	657.80	*ud*	13.07	.11	420.2	27171.62
AB08	111.40	*ud*	920.57	3730.29	5059.95	*ud*	9.22	1.69	657.8	20143.55
AB10	307.21	2.20	114.23	474.90	870.25	*ud*	14.53	*ud*	5059.95	16640.56
AB11	131.65	*ud*	978.75	2647.08	3682.55	*ud*	11.73	*ud*	870.25	16693.92
AA01	228.02	*ud*	-	-	-	.60	-	2.03	3682.55	-
AA02	103.85	*ud*	-	-	-	1.02	-	1.37	10127.05	-
AA04	-	-	-	69.63	420.20	-	413.24	-	706.1	23135.39

Note. – indicates no data available

Note. ud indicates the concentration was below detection range of Enzyme Linked Immunosorbent Assay kit used for analysis

Abbrv. CTX-II C-Terminal Cross Linking Telopeptide of Type II Collagen, *IL-6* Inrerleukin-6, *PIICP* Procollagen Type II Collagen Propeptide, *sf* synovial fluid, *u* urine.

CHAPTER 7: GENERAL DISCUSSION

The aim of my Master's Thesis was to determine the most commonly used biomarkers of knee joint healing, and to measure these biomarkers in adolescents with ACL injuries. To address this aim, two research questions were (Q1) Based on the existing literature, which biomarkers are the most commonly used when identifying knee joint healing in patients with ACL injuries, and what is the age of these study participants? (Q2) What is the association between concentrations of a) sfCTX-II and uCTX-II, b) sfIL-6 and uIL-6, and c) sfPIIC: sfCTX-II and uPIICP: uCTX-II, in adolescent patients with ACL injuries?

7.1 Based on the existing literature, which are the most studied biomarkers reflective of knee joint healing in patients with ACL injuries?

We hypothesized that we would conclude from this systematic review that CTX-II and IL-6 will be the most common biomarkers used to assess healing in patients with ACL injuries (H1a; Bellido et al., 2011; Bigoni et al., 2016; C. et al., 2018; Chmielewski et al., 2012; Delmas, 2009; Friel et al., 2013; Wang et al., 2020; Lotz et al., 2013). We also hypothesized that participant ages in the included studies for this systematic review will also be adult (H1b; Harkey et al., 2015). Based on the findings of our systematic review, we determined that there is very limited existing research and evidence on the use and findings with respect to biomarkers and healing status post ACL injury and surgical reconstruction. Our review found: (i) only six studies evaluated physiological healing using biomarkers following an ACL reconstruction, (ii) uCTX-II and sfIL-6 were the most studied biomarkers, and (iii) there is a significant literature gap with respect to evaluating biomarkers of knee joint healing in adolescents following an ACL reconstruction.

ACL injuries are complex and can impact physiological, biomechanical and psychosocial components (Kvist et al., 2018). Therefore, research on ACL injuries used to guide rehabilitation should consider these three aspects. In this review we have focused on the physiological component, and the lack of research on adolescents. Further research with adolescent participants is necessary to build the scientific foundation of biomarkers of the healing process in the knee joint. This should include multiple variables assessing inflammation (e.g. sfIL-6), cartilage health (e.g. uCTX-II), and synthesis (e.g. PIICP). Adolescents are unique from adults due to growth (Kronenberg, 2003) and sex hormone variation, indicating that biomarker research in adults should not be extrapolated to adolescents (Juul, 2001). Therefore, (i) more research is needed specific to adolescents and, (ii) this research should explore uCTX-II and sfIL-6 since they are the most studied biomarkers reflecting knee joint healing.

7.2 What is the association between concentrations of local synovial fluid and systemic urinary biomarkers of healing in adolescent patients with ACL injuries?

The purpose of this study was to be the first to examine the feasibility of examining the associations between local synovial fluid, and systemic urine samples of biomarkers of knee joint healing. To address this, we posed the following research questions: in adolescent patients with ACL injuries, what is the association between concentrations of a) sfCTX-II and uCTX-II, b) sfIL-6 and uIL-6, and c) sfPIICP: sfCTX-II and uPIICP: uCTX-II? This pilot study provided direction for further data analysis in order to fully answer these research questions. This retroactively modifies the aim of this study as the results from this study reveal a research question that should be evaluated prior to investigating local and systemic associations of these biomarkers. We revealed that the optimization of sample preparation and assay conditions must first be thoroughly addressed and we could therefore rephrase this intermediary research question

as: What is the optimal methodology in order to produce reliable results of urine and synovial fluid levels of IL-6, CTX-II and PIICP in adolescents with ACL injuries?

Synovial Fluid Analysis

Synovial fluid analysis is challenging due to its complex matrix and viscous non-homogenous matrix (Fam et al., 2007). Methods to reduce synovial viscosity like dilution and/or hyaluronidase (HAse) treatment are routinely used before a variety of analytic techniques (Song et al., 2012; Mateos et al., 2012; Sugiuchi et al., 2005; de Jager et al., 2007; Ekmann et al., 2010). Jayadev et al. conducted a study motivated by their own low and variable repeatability of cytokine measurements in neat synovial fluid samples from patients with knee OA (Jayadev et al., 2012), similar to what we have reported in Chapter 6. Their samples for analysis by Luminex bead analysis were treated with i) no preparation (neat), 1:2 dilution, 1:4 dilution, or treatment with 2 gm/mL HAse. They concluded that the increased dilution factor produced more precise biomarker concentrations compared to no treatment, and the treatment with HAse resulted in the most improved measured signal and recovery of target analytes in their bead-bead Luminex assays. This study had a sample size of 7, and given our similar dilemma and sample size, a similar technical paper with the data presented and with further research using HAse digestion could provide insightful information for further research to address the purpose of this thesis, and to inform others using a similar methodology.

Urine Analysis

Unexpectedly, urine levels of CTX-II and PIICP were much lower than expected when compared to existing literature, and under the detection range in some samples. There is currently debate over the preferential methodology when it comes to urine collection and

analysis for biomarker concentration. “Urine contains over 1500 extracellular and membrane bound proteins, cells, inorganic ions (K+, Na+, Cl− and Ca2+) and organic molecules like creatinine, urea, and uric acid. All these substances can hinder the efficient binding of a protein to its corresponding antibody in an ELISA assay (Adachi et al., 2006). Variability of urine matrix components including electrolytes or pH can also impact the performance of the assay (Fichorova et al., 2008). The correction for creatinine is one way to account for the biological complexity of this sample. This complexity is largely why there are limited clinical applications of urine testing at this time, due to lack of validation on a clinical level (Chatziharalambous et al., 2016).

In order to improve upon the reliability of the biomarker concentrations in urine, aspects of sample collection and analysis must be addressed. Samples could be collected earlier in the morning with first or second void urine in order to increase the concentration of the urine which may lead to biomarkers in the detectable range of the current assay. To improve upon assay sensitivity, samples could be analyzed in triplicates as opposed to duplicates. In addition, future analyses could evaluate samples using a western blot given the increased sensitivity of this method (Karlsson et al., 1989), or simply repeating this analysis on various commercially available ELISA kits for urine biomarkers. Diluting samples from a range of 1:2 to 1:60 as performed by Chmielewski et al. should also be explored given their methodology yielded high and detectable concentrations of this biomarker in uninjured controls. A diluent of phosphate buffered saline has been recommended for urine samples (Chatziharalambous et al., 2016). Collecting and analyzing plasma levels as an additional systemic biomarker could also prove relevant to confirm the level of this biomarker in the systemic urine samples.

7.4 Conclusion

The overall objective of this thesis was to explore biomarkers of knee joint healing in an adolescent population with ACL injuries. The motivation behind this research is that the rates of ACL injuries are continuing to rise in adolescents. This puts them at risk for developing knee OA, and this risk increases when they are prematurely cleared for return to activity following ACL reconstruction surgery. ACL injuries are complex and can impact physiological, biomechanical and psychosocial components (Kvist et al., 2018). Therefore, research on ACL injuries used to guide rehabilitation should consider these three aspects. In this thesis we have focused on addressing this physiological component of ACL injuries and recovery, and we able to provide basis for future direction to continue research in this area starting with studying reliability of biomarker measurements, feasibility of systemic measures, and analysis of these biomarkers over a longitudinal post-operative study period. This will hopefully lead to an impact on clinical practice, and reducing the risk for knee OA in adolescents following ACL reconstructions.

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