The Association Between Serum COMP Expression and Intra-articular Knee Injury Sustained 3-10 Years Previously in Youth Sport.

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The Association Between Serum COMP Expression and Intra-articular Knee Injury Sustained 3-10 Years Previously in Youth Sport.

by

Jordan Laudon

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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ABSTRACT

Purpose:

This research investigates the relationship between knee injury and cartilage oligomeric matrix protein (COMP), a known indicator of joint deterioration in osteoarthritis (OA).

Methods:

170 participants were recruited aged 16-26 years (85 with a sport-related intra-articular knee injury 3-10 years ago and 85 age, sex, and sport-matched controls). Serum samples were collected to evaluate COMP levels before and after physical activity.

Results:

Male participants averaged 15% greater (219.5 ng/ml, 95% CI 11.0–428.0, p = 0.04 serum COMP levels than uninjured controls. Female participants showed no significant elevation (9.8 ng/ml, 95% CI -208.4-227.9, p = 0.93). There were no differences between injured and uninjured participants in change in COMP pre to post exercise.

Conclusions:
This research demonstrated differences in baseline COMP values between participants based on history of knee injury in males only. Future research into specific knee injuries may yield insights in COMP’s value identifying risk of OA.
Acknowledgements

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I would also like to acknowledge my supervising committee, Dr. David A. Hart, Dr. Linda Woodhouse, and Dr. Jackie Whittaker, who have provided a tremendous amount of guidance in producing quality research.

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<tr>
<td>ADAMTS</td>
<td>A Disintegrin And Metalloproteinase With a ThromboSpondin Type-1 Motif (aggrecanase)</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index (kg/m(^2))</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage Oligomeric Matrix Protein</td>
</tr>
<tr>
<td>hCOMP</td>
<td>human Cartilage Oligomeric Matrix Protein</td>
</tr>
<tr>
<td>sCOMP</td>
<td>serum Cartilage Oligomeric Matrix Protein</td>
</tr>
<tr>
<td>ΔsCOMP</td>
<td>Change in Serum COMP due to physical activity (Post-Exercise - Pre-Exercise)</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual X-ray Absorptiometry</td>
</tr>
<tr>
<td>FMI</td>
<td>Fat Mass Index (kg/m(^2))</td>
</tr>
<tr>
<td>IU/l</td>
<td>International Units/Liter (arbitrary measure)</td>
</tr>
<tr>
<td>JSW</td>
<td>Joints Space Width</td>
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<tr>
<td>KOOS</td>
<td>Knee Osteoarthritis Outcomes Score</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>Par-Q</td>
<td>Physical Activity Readiness Questionnaire</td>
</tr>
<tr>
<td>PTOA</td>
<td>Post-traumatic Osteoarthritis</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>Tumor Necrosis Factor Alpha</td>
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<tr>
<td>VAS</td>
<td>Visual Analog Scale</td>
</tr>
<tr>
<td>WOMAC</td>
<td>Western Ontario &amp; McMaster Universities Osteoarthritis Index</td>
</tr>
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1. BACKGROUND:

For decades, biomarkers have been used as a method to identify and assess the presence and progression of a variety of diseases. To date, no single biomarker has been found that predicts the presence of osteoarthritis (OA). However, preliminary investigations suggest that cartilage oligomeric matrix protein (COMP) may have prognostic value for worsening OA.\textsuperscript{1,2} To date no investigation has identified a biomarker with prognostic value for diagnosis of OA prior to disease onset. Serum COMP (sCOMP) may prove to be of prognostic value in regards to early diagnosis of OA. More recently, changes in sCOMP (ΔsCOMP) have been identified in response to physical activity. The magnitude of ΔsCOMP is known to modulate based on previous training activities,\textsuperscript{3} but is poorly understood in general.

1.1 Purpose

OA is understood to be a pathological degenerative joint condition defined by the presence of pain, reduced mobility, and radiographic changes including cartilage loss, joint space narrowing, and osteophytes.\textsuperscript{4,5} While OA most typically develops in older populations, post-traumatic osteoarthritis (PTOA) has been consistently identified in young to middle-aged adults 12-20 years post injury.\textsuperscript{6} Knee injury is one of the most common sport related injuries in youth and PTOA is most commonly described in the knee joint.\textsuperscript{7} In Canada, sport participation is the leading cause of injury in youth, with knee and ankle injuries accounting for over 40% of the burden.\textsuperscript{8} Furthermore, studies indicate a 10-fold increased risk of knee OA in the 12-20 years
post injury.\textsuperscript{8} It is estimated that over 50 percent of individuals with an anterior cruciate ligament (ACL) tear or meniscal injury will develop knee OA.\textsuperscript{9}

Presently, there is preliminary research examining sCOMP outcomes immediately following intra-articular knee injury or after PTOA onset.\textsuperscript{10,11,12} However, there is a paucity of research examining outcomes during the interval between joint injury and disease onset (<10 years post-injury). Furthermore, no research has investigated if $\Delta$sCOMP as a potentially viable biomarker for PTOA. The purpose of this research is to evaluate the potential role of COMP and $\Delta$sCOMP as biomarkers to detect and predict early PTOA in an at risk population in comparison to healthy controls.

1.2. Background

COMP is a structural protein integral to proper articular cartilage function. Studies have implicated COMP as a prognostic indicator of worsening OA within previously diagnosed individuals. Increasing levels of COMP have correlated to subsequent radiographic degradation of articular surfaces in the knee.\textsuperscript{1,2} To date, few researchers have investigated COMP in young adults (16-26 years) or in relation to PTOA.\textsuperscript{13} Given the potential utility of COMP as a diagnostic and prognostic indicator of OA development, there is a notable age gap in published literature concerning COMP and early OA development. Published studies tend to focus on juvenile (under 18 years) and older (45+ years) populations. COMP levels have been identified as a surrogate measure of articular turnover rates,\textsuperscript{14,15,16} and are strongly implicated in fibrillogenesis,\textsuperscript{17} a process integral to maintaining a healthy mechanical within articular cartilage.
Furthermore, sCOMP has been shown to fragment in uniquely different patterns depending on the type of disease afflicting the joint. Categorical differences between rheumatoid arthritis (RA) and OA have been shown in the literature. Such differences may be present during the years prior to clinical OA diagnosis within afflicted serum.

1.3. **Rationale**

Very little is known about sCOMP levels in adolescent populations. While sCOMP has been studied extensively in older populations (greater than 45 years of age), little is known about sCOMP characteristics in younger age groups. In older populations, sCOMP has demonstrated value as a prognostic indicator of OA progression in knee joints. The potential prognostic value of sCOMP has not been investigated in younger populations at risk of developing PTOA.

The association of sCOMP values at rest in relation to a history of intra-articular knee injury is an area that has not been investigated to date. It is known that intra-articular injuries increase the risk of OA within the injured joint, but the potential relationship to sCOMP and ΔsCOMP is unclear. The potential relationships between sCOMP, ΔsCOMP, and OA development are of tremendous research interest. Knowledge surrounding the potential associations between OA and sCOMP and ΔsCOMP levels could provide the basis for identifying participants at an increased risk of developing PTOA. Early identification would allow for secondary intervention strategies whose effectiveness would be diminished substantially by a delayed identification of PTOA risk or presence.
Adiposity and sCOMP levels have been investigated in cases of morbid obesity, and participants over the age of 45. Both investigations found a positive association between body mass index (BMI) and sCOMP. However, the potential association between the two has not been investigated in milder cases with relatively healthy participants. As obesity and COMP have been linked in literature, it is prudent to investigate this potential association within the confines of this research.

To date, no research has investigated the potential relationships between COMP, ΔsCOMP, and the self-report Knee Injury and Osteoarthritis Outcome Score (KOOS). Of particular interest are the KOOS symptom sub-scale score. The KOOS questionnaire is very simple to apply, taking only a few minutes to fill out. A significant association between KOOS and sCOMP or ΔsCOMP could provide an economical way to decide whether at risk individuals ought to receive additional testing (e.g., sCOMP levels).

This study will investigate sCOMP levels in individuals who are at an increased risk of developing PTOA 3-10 years after an intra-articular knee injury in comparison to age, sex and sport matched healthy controls. Elevated COMP values may prove to be an important indicator of which participants are at highest risk of developing OA.

1.4. Research Questions

Are there differences in sCOMP and ΔsCOMP between individuals with and without a 3-10 year history of intra-articular knee injury sustained during youth sport? Furthermore, are sCOMP and
ΔsCOMP levels influenced by adiposity (fat mass index; FMI) and KOOS symptom sub-scale score.

1.5. Primary Objectives

The primary objective of this study is to examine the association between history of sport-related intra-articular knee joint injury sustained in youth (≤ 18 years) and expression of sCOMP and ΔsCOMP. Higher sCOMP values would imply greater amounts of articular turnover at rest, which may be indicative of future joint degeneration. Higher values of ΔsCOMP would imply a larger increase in turnover, implying that a greater strain has been placed on the articular environment within the knee.

Hypothesis 1: There will be an association between history of intra-articular knee joint injury (sustained 3-10 years previously) and baseline (pre-exercise) sCOMP expression. Specifically, participants with a history of knee injury will demonstrate a significantly different baseline (pre-exercise) sCOMP expression compared to uninjured age, sex and sport matched controls.

Hypothesis 2: There will be an association between history of intra-articular knee joint injury (sustained 3-10 years previously) and ΔsCOMP expression. Specifically, participants with a history of knee injury will demonstrate a significantly different ΔsCOMP values than uninjured age, sex and sport matched controls.
1.6. Exploratory Objectives

Exploratory objectives include evaluating the association between COMP outcomes (Pre-Exercise COMP and ΔsCOMP) to FMI and the KOOS symptoms sub-scale score.

Hypothesis 3: Increasing self-reported knee symptoms, as measured by the KOOS symptom sub-scale, will be associated with greater increases in ΔsCOMP expression in injured and uninjured participants. More symptomatic knees may be more prone to inflammatory responses to physical activity, leading to a spike in sCOMP values in serum.

Hypothesis 4: Increasing FMI will be associated with greater increases in ΔsCOMP expression in injured and uninjured participants. Increasing FMI is likely to place additional mechanical strain on the joint as well as contributing to a more inflammatory environment within the joint. Combined, these effects may contribute to elevated ΔsCOMP outcomes.

Hypothesis 5: Increasing self-reported knee symptoms, as measured by the KOOS symptom sub-scale will be associated with greater levels of pre-exercise COMP expression in injured and uninjured participants. More symptomatic knees are likely in more inflamed states, leading to an increase in articular turnover.

Hypothesis 6: Increasing FMI will be associated with greater levels of pre-exercise sCOMP expression in injured and uninjured participants. Greater FMI may result in increased mechanical
strain and a more inflamed resting state. Both these factors could lead to increased pre-exercise values of sCOMP.

Hypothesis 7: Differences in age, sex, sCOMP, and ΔsCOMP may be associated with different distributions of sCOMP fragmentation. Differences have been identified between different arthroses, but are largely unexplored.

Significant associations between FMI and baseline sCOMP would confirm findings within the literature. Associations between FMI and ΔsCOMP would represent new findings related to sCOMP, and substantially further the understanding of the underlying contributors to ΔsCOMP values. Associations identified between KOOS symptom sub-scale score, sCOMP, and ΔsCOMP would also represent novel findings within the field. Differences in sCOMP fragmentation or ΔsCOMP fragmentation would be the first of its kind. Confirmation of many of the exploratory outcomes would represent unique findings relating to sCOMP and ΔsCOMP.
2. LITERATURE REVIEW

2.1. Post-traumatic Osteoarthritis

It is estimated that 9.8% of all OA cases are associated with a prior traumatic event.\textsuperscript{11} Knee joint trauma may include sprains to the cruciate and collateral ligaments, meniscal tears, intra-articular fractures, patella-femoral subluxations and dislocations.\textsuperscript{23} These are often associated with concomitant lesions involving the synovium, articular surface and subchondral bone.\textsuperscript{24,25} Meta-analyses indicates a 3.86\textsuperscript{26} (95% CI 2.61-5.70) fold increased risk of developing OA after significant trauma to the knee joint.\textsuperscript{9} This phenotype of OA is referred to as PTOA and unlike idiopathic OA, which is usually associated with adults between the ages of 60-80 years, PTOA is associated with an earlier onset with symptoms presenting before 30 years.\textsuperscript{12}

A study from Johns Hopkins of 1,321 former medical students found that among those who suffered a traumatic knee injury, 13.9% had OA by 65 years, compared to 6% among those who did not [relative risk, 2.95 (95% CI, 1.35 to 6.45), p=0.0045].\textsuperscript{27} Current evidence indicates that an increasing number of individuals develop PTOA as a result of knee injuries in their youth.\textsuperscript{12} Sport and recreation account for >30% of all injuries sustained in youth with knee injuries being among the most common.\textsuperscript{7,28,29,30} While previous joint injury has been associated with the onset of OA, the pathogenesis of OA is complex, and multiple risk factors must be considered.\textsuperscript{12,31,32}

2.2. Cartilage Oligomeric Matrix Protein
Cartilage oligomeric matrix protein (COMP), or thromospondin-5 is a possible PTOA disease marker. COMP, a pentameric 535kDa (kilodaltons) noncollagenous extracellular matrix protein expressed within articular cartilage, is of particular interest because it is accepted as a relative indicator of overall cartilage turnover.\textsuperscript{14,15,16} COMP has been shown to play a role in fibril formation of collagens I and II by promoting early association of collagen molecules, thereby accelerating and mediating fibrillogenesis, influencing the distinct organization of the fibrils.\textsuperscript{33} In addition, COMP regularly binds to heparin, chondroitin 4 sulfate, chondroitin 6 sulfate, and aggrecan within articular cartilage.\textsuperscript{34} The interaction of aggrecan with the collagen fibril meshwork organized by COMP produces the structure necessary to resist articular compression.

In addition to binding to many structural proteins in cartilage, COMP has demonstrated binding to fibronectin in a dose dependent manner.\textsuperscript{8} Fibronectin is a glycoprotein known to interact with integrin, a trans-membrane protein expressed in all chondrocytes. Trans-membrane proteins are lodged in the cell surface, and can be active on either side of this barrier. This interaction between integrin and COMP, mediated by fibronectin, suggests that varying levels of COMP may produce differing local effects on chondrocytes, the articular structure and turnover rates within cartilage. Fibronectin is thought to be integral to cell adhesion within the extracellular matrix.\textsuperscript{35} To date the effects of fibronectin binding on cellular activity remain largely unknown in regards to OA.\textsuperscript{36}

While COMP has the ability to interact with a variety of structural (ECM) and intercellular proteins, it is known to regulate by a number of mechanisms. One of the main regulators at the protein levels are the matrix metalloproteins (MMP’s) 3, 12, 13, and ADAMTS 4,5 (aggrecanase
These proteins are all linked to inflammatory pathways and all have demonstrated the capability to degrade extracellular COMP. COMP fragment release is also known to be stimulated by mechanical compression of articular tissues. The magnitude of the change in COMP in response to mechanical compression, and its determining factors, are not well understood.

Mutations within COMP can lead to severe complications, the most common of which is pseudoachondroplasia which is the result of misfolded COMP proteins. Symptoms include short stature, small hands and feet, as well as joint abnormalities and early onset OA. Together, these studies suggest that COMP plays an integral role in mediating molecular interactions in cartilage and is critical to the proper load-bearing function of cartilage. While the pathways highlighted here are the most significant and investigated connections to COMP, they are by no means the only established interactions. COMP has connections to a variety of proteases, cellular pathways, growth factors, and extracellular matrix proteins.

2.2.1. COMP Measurement in Healthy Individuals and Individuals with Osteoarthritis

To date there is no consensus on normal baseline COMP expression in the healthy or OA population however it appears that sex, race, age and BMI all play a role. For example, investigators of the Johnston County OA (North Carolina, USA) project revealed that Caucasian males have increased baseline COMP expression in comparison to Caucasian females. Meanwhile, African-American females have increased baseline expression in comparison to males. In addition, these investigators found a positive correlation between baseline COMP
expression, BMI, and weight ($r=0.14$, $r^2=0.02$, $p=0.0001$ for both BMI and weight) and an elevated expression in COMP associated with a diagnosis of OA in both sexes, and across all age groups (45-54, 55-64, and 65-85).\(^1\) These findings indicate the importance of considering sex, race, BMI and age in COMP analyses. To our knowledge, there have been few studies of COMP expression in younger or adolescent populations similar to the age range of this study. Thus, any conclusions drawn from these studies are limited in their generalizability to the current study.

Investigations into the diurnal variations of sCOMP levels have revealed that in healthy, OA, and RA populations aged 35-78 years, values remain relatively constant throughout the day.\(^{10,43,44}\) sCOMP values have been shown to drop during bed rest in arthritic and healthy controls, with a lowest sCOMP value reached between 4:00-7:00 a.m.\(^{10,43,44}\) This can be explained by sCOMP’s estimated half-life in the blood of 7.4 hours and the relative lack of joint loading during sleep.\(^{10}\) COMP samples collected after waking and light joint loading activities of daily living (e.g., showering, morning routine, and non-strenuous activity) returned to the constant levels seen throughout the day. This is consistent with other studies that have demonstrated an increase in COMP after even light activities.\(^{20}\) Concentrations during all other waking hours remained relatively constant.

2.2.2. **Effects of severity of osteoarthritis on COMP**

Recent work has demonstrated that sCOMP expression after 30 minutes of exercise correlates with cartilage thickness in female OA patients 40 years and older over a 5-year period.\(^{45}\) Further, COMP has been shown to be a rough diagnostic indicator of radiographic OA presence and
severity.\textsuperscript{1,46,47} However, considerable variation of baseline expression has limited its prognostic utility. The Johnston County OA Project found baseline levels of sCOMP were elevated in participants with OA aged 45-85 years and increases in expression correlated closely with the radiographic progression of OA measured by the Kellgren-Lawrence radiographic scale and the number of OA afflicted joints.\textsuperscript{1,48} This is to be expected, as COMP expression is not exclusive to knee cartilage and increased matrix turnover is likely to occur in any joint afflicted by OA. This study also found a significant increase in COMP expression in OA patients over age 65 in comparisons with patients aged 45-65 years in both sexes.\textsuperscript{43} An explanation for this increase was not put forth by the authors, but it could be a reflection of the worsening articular condition associated with age or perhaps a change in the half-life for COMP in serum. COMP half-life in serum was predicted as 7.4 hours in participants aged 60-64, but is unknown in other age groups.\textsuperscript{10}

Reported sCOMP levels were higher in patients with bilateral versus unilateral symptomatic hip OA (age 56.4 +/- 14.1).\textsuperscript{49} Increased sCOMP levels have also been associated with bone scan abnormalities, suggesting that serum concentrations reflect changes in the tissue turnover seen on bone scans.\textsuperscript{15} In older patients with clinically and radiographically diagnosed OA (aged 56.4 years with a standard deviation of 14.1 years), the change in joint space width of both knees over 3 years correlated positively with sCOMP levels.\textsuperscript{50} Hip OA patients who progressed by at least two Kellgren-Lawrence grades on their radiographs were shown to have significantly higher COMP levels at baseline and at a one year follow-up, indicating a link between elevated COMP and progressive OA pathology. A shift of two K-L grades represents a substantial worsening of joint condition. In knee OA, sCOMP expression correlated with the clinical manifestation of
synovitis, but did not correlate with the extent of joint damage. This suggests that sCOMP elevation may indicate a state of OA progression, but is not an indicator of the severity of articular damage.

In a study by Richette et al which examined morbidly obese knee OA patients (n=44, mean age 44 years with a standard deviation of 10.3 years), gastric surgery was performed with the aim of reducing adiposity and OA symptoms. Six months post-surgery, the average BMI dropped from 50.7 kg/m² to 40.4 kg/m² (test statistic, p<0.0001). Pain, based on a 100mm visual analog scale (VAS), decreased by 51% (p<0.0001). WOMAC scores (Western Ontario and McMaster University Osteoarthritis Index, a clinically verified measure of symptoms in hip and knee OA) significantly improved on pain (-50%, p<0.0001), stiffness (-47%, p<0.0001), and function (-57%, p<0.0001) subscales. COMP expression decreased from 10.5 +/- 3.5 IU/l (international units/liter, an arbitrary value) to 6.7 +/- 2.2 IU/l (p< 0.001) and levels of insulin decreased from 15.4 +/- 9.6 IU/l to 8.7 +/- 6.6 IU/l (p <0.0001).

COMP release is known to be mechanosensitive in vitro. Given the link between weight loss and reduced joint load, much of the reduction in COMP levels is likely related to sheer weight loss. However, the potential effects of reduced systemic inflammation in response should not be discounted. A combination of reduced weight and systemic inflammation may produce a complementary reduction in inflammation within specific joints.

2.2.3. COMP and Diagnostic Imaging Evidence of Degeneration in OA
Magnetic resonance imaging (MRI) is a medical imaging technique that allows for the visualization of the anatomy and physiology of internal structures. With respect to knee joint OA, cartilage thickness, joint space width (JSW), articular lesions, and osteophytes can all be visualized. A common tool in diagnostics, traumatic knee injuries have correlated to specific structures visualized on MRI’s of the knee. While elevated COMP has been associated the development of OA, some features of MRI assessments have correlated exceptionally well with elevations in baseline COMP. In particular, osteophyte development and joint-space narrowing within the intra-articular space have been demonstrated to have positive associations with elevated COMP. Both of these features have been associated with OA. Meta-analysis suggests that post-ACL surgery, macroscopic changes to the articular surface often occur within two years of the injury. In particular, increased COMP expression has been implicated as an incident to three-year predictor of osteophyte formation, but not an effective three to six year predictor of osteophyte formation. This suggests that COMP may be a predictor only in early stage OA.

2.2.4. COMP Levels in Response to Physical Stress

It is known that COMP expression is modulated after moderate amounts of physical activity. For example, Andersson et al demonstrated a significant increase in median COMP values immediately following 60 minutes of high intensity (>60% Max heart rate) exercise 10.5 UI/l to 11.6 UI/l (p=0.018) in patients ages 36-65 years with knee OA. The increase at 90 minutes was no longer significant, although reported values remained elevated over 6 hours post-exercise. The loss of significance may be a function of a small sample size (n=7) rather than an indicator of changes in elevated status. Another study of medial knee compartment OA in participants...
ages 40-74 years (n=42) demonstrated a significant elevation in COMP immediately after a 30 minute walking test (6.3% increase, p<0.001 ).\textsuperscript{21} Thirty minutes post exercise completion, COMP values were not discernible from baseline values (p=0.009). A study of COMP expression in healthy young adults (25-34 years) during noncompetitive marathon events found that baseline COMP expression was significantly elevated compared to controls and did not return to baseline until 24-48 hours post marathon.\textsuperscript{61} It is not clear how much of the difference in COMP expression shift is due to exercise intensity, physiology, and/or age. The authors suggest that inflammation may also play a role in the continued elevation of COMP post exercise, a view substantiated in the literature.\textsuperscript{61,62} This suggests that chronic exercise, such as marathon training, may have a lasting effect of elevating baseline COMP levels as a function of the inflammation induced by intense training. The correlation found between sCOMP and inflammatory mediators TNF-alpha and IL-1RA\textsuperscript{61} suggest a link between lasting sCOMP elevation (24-48 hours), intense physical training, and inflammation. Acute bouts of exercise, in comparison, produce much more transient elevations in sCOMP levels.\textsuperscript{60}

COMP is present in a number of tissues throughout the body, but it is believed that mechanical stimulation transfers articular material to the synovial fluid, where it can be drained into the lymphatic system and into the blood supply. A study by Niehoff et al compared blood sCOMP expression after a variety of loading protocols in healthy young males including rest, deep knee bends, lymphatic drainage, and running.\textsuperscript{63} As expected, 30 minutes of rest induced a significant decrease in sCOMP. Lymphatic drainage and knee bends did not produce any significant changes. However, 30 minutes of running induced a significant increase in sCOMP that persisted up to 90 minutes post-intervention. These results suggest that the increases in sCOMP induced
by running are induced by more than fluid cycling (lymphatic drainage) or cartilage deformation (knee bends). Alternatively, COMP (and/or COMP fragments) may be aspirated by repetitive articular compression, with a significant weight-bearing load, at a high frequency (running).\textsuperscript{64}

Another study demonstrated a reduction in this transient increase in response of sCOMP expression to a 30 minute walking test, in the form of a jogging intervention program. Cycling and swimming interventions of equal cardiovascular strain did not reduce the magnitude of the transient shift in COMP expression compared to untrained controls.\textsuperscript{3} This suggests that COMP levels may be modulated by a functional adaptation within the cartilage. Previous exposure may change the physical attributes of the cartilage, resulting in less COMP release. This may be a meaningful adaptation, or simply a change in the rate of COMP being transferred from cartilage into the synovial fluid, or to the serum where it is sampled. Much remains unknown about COMP expression in response to exercise, and the relevance of functional adaptations of COMP expression.

\subsection{COMP Measurement}

COMP exists in two forms; whole and fragmented. However, depending on the type of fluid sample used, different sCOMP forms are likely to be identified. Samples of synovial fluid typically contain both whole and fragmented isoforms of COMP. In synovial fluid, those with degenerative joint disorders such as OA and RA tend to show greater fragmentation of COMP. Blood serum samples, on the other hand, mostly contain COMP fragments sized 50-90kDa.\textsuperscript{65} It is unclear precisely where COMP is degraded from its intact form into fragments. While most
studies do not distinguish between the forms, there is evidence to suggest the difference may have biological significance. In one recent study, sCOMP expression was compared between OA, RA, and healthy control participants. While traditional ELISA methods produced similar expression levels in all patients, a novel COMP capture method exclusively for COMP fragments showed significant elevation of MMP-13 and ADAMTS-7 COMP fragment expression in OA patients. Furthermore, the increase in COMP fragments correlated closely with Kellgren-Lawrence ratings of radiographic arthritic progression in the OA participants. To date, there are twelve definitively identified neoepitopes of COMP. One epitope, S, is a 45KDa fragment that has been identified in synovial fluid from patients with OA and RA, as well as immediately after knee trauma. Specifically, this fragment has been tied to exposure to TNF-α and IL-6 (Tumor necrosis factor-alpha and interleukin-6, catabolic proteins expressed in articular cartilage). Knowledge of specific fragment lengths may lead to additional identifications of individual catabolic processes resulting in specific COMP fragments. To date, however, the distinction between fragmented and intact protein has not been investigated in relation to age, adiposity, or physical activity.

It should be noted that COMP has not yet been accepted as a diagnostic indicator of OA in North America. While correlations between COMP expression and OA exist, the evidence does not support current whole COMP measurement for diagnosing OA. The COMP assay kit used in this study is approved for identifying cartilage turnover in patients within the European Union, but it has not been approved internationally.
2.4. Osteoarthritis and Blood Serum Biomarkers

To date, no early OA biomarkers investigated have produced diagnostic assays in North America, although COMP has been recognized as an “in-vitro diagnostic device” of future joint destruction within the European Union. This is partly due to the amount of potential variability in a given biomarkers expression within the normal and OA populations. Historically, single biomarkers diagnostic assays have been proposed. However, these assays have not been widely adopted due to the absence of a unique, ubiquitous marker of OA that is differentially regulated (absent vs. present) between the normal and disease states. Furthermore, while some biomarkers have been associated with OA, no single marker has demonstrated a significant predictive value for OA or PTOA. The pathophysiology of PTOA progression is not well understood, particularly in relation to biomarkers. To date, there has been little study of potential effects of biomarkers on PTOA development in youths age 16-26.

Consideration of blood serum biomarkers in the period between joint injury and PTOA onset may provide insight into underlying disease mechanisms and lead to the identification of early diagnostic assays. The difference between normal degradation with aging and the degradation involved in OA has not been clearly delineated at this time.

2.5. Etiology of Post-traumatic Osteoarthritis

While knee OA is often considered a disease of age, PTOA is a distinct subgroup within the population affected by OA predicated by prior significant trauma to the knee joint. While the
etiologic pathway for PTOA is not fully understood, it is believed to be initiated by injury which results in a combination of changes in joint biomechanics and long term inflammatory environment of the joint.\textsuperscript{47,52,58,59}

In addition to damage to tissues surrounding the articular cartilage, PTOA can also develop from damage directly to the articular surface, as disruption of extracellular matrix is a significant risk factor for PTOA. As such, tibial fractures may increase the risk of PTOA up to 20-fold, and often induce PTOA in as little as two years.\textsuperscript{38} Fractures of greater size and magnitude have been demonstrated to have worse outcomes at follow-up than minor fractures.\textsuperscript{38}

2.6. Adiposity and Osteoarthritis

A number of studies have reported associations between BMI and the incidence of OA. OA of the knees and ankles may be related to excess weight.\textsuperscript{47} A British study found that females in the highest tertile of BMI had an odds ratio of 6.17 (95% CI 3.26-11.71) for knee OA, and an odds ratio of 17.99 (95% CI 6.25-51.73) for bilateral knee OA in comparison to the lowest third of BMI.\textsuperscript{22} In a longitudinal study of males and females aged 40-64 years, Manninen et al reported that for every standard deviation increase of BMI (3.8 kg/m\textsuperscript{2}), there was a 40% increase in risk of developing knee OA.\textsuperscript{33} In addition, BMI was also associated with OA of non-weight bearing joints,\textsuperscript{47} such as the wrists and fingers, suggesting that obesity may contribute to OA in ways other than increased load in the joints, such as the production of inflammatory cytokines.
In these studies, BMI was the primary adiposity outcome. However, BMI has some notable limitations as an outcome of adiposity in that it provides no distinction between lean tissue and fat mass. Fat mass, particularly visceral adipose tissue, has been tied to increased incidence of cardiovascular disease and diabetes.\textsuperscript{40, 69} One study from the Netherlands in males and females aged 45-65 years found Visceral Adipose Tissue (VAT) content to be a risk factor for hand OA, providing further evidence that OA is a risk factor for non-weight bearing OA.\textsuperscript{70} To our knowledge, there has been no study comparing knee OA incidence and VAT content. Due to the connection of various systemic disorders to VAT content, and the imprecise nature of BMI at the individual level, dual x-ray absorptiometry (DXA) was used in this study to determine FMI, which has been demonstrated to be an accurate predictor of total VAT in overweight populations when measured by DXA ($r^2 = 0.86$) in comparison with CT scans.\textsuperscript{84} A study from 2012 demonstrates the improvements of DXA evaluations of VAT, demonstrating a strong coefficient of determination across all ages, sexes, and adiposity levels (combined $r^2 = 0.957$).\textsuperscript{85}

\section*{2.7. Sex Differences in Osteoarthritis}

While rarely the a priori subject of investigations, substantial differences in the incidence and progression of OA occur based on sex. Females are more likely to injure their ACL’s during sport, and have a marked increase in the prevalence and progression of OA after menopause.\textsuperscript{54} This elevated risk of ACL injury is reflected in the distribution of injuries by sex, shown in the distribution of injury types among study participants in the results section. Furthermore, overall prevalence of OA in the United States is approximately 40\% higher in females than males, with higher prevalence across all age ranges from 18-84 years.\textsuperscript{71} Males and females also react
differently to OA, with females reporting more substantial reductions in quality of life and function than males. While OA pain can be related to the innervation of the knee joint, it does not always correlate with injury severity and often persists even after total joint replacement. Mechanisms underlying these differences in pain between sexes with regards to knee OA are not known. In addition, differences in articular chondrocyte behavior and metabolism, including sex-hormone receptor frequency and hormone responses have been associated with phases of the menstrual cycle. These sex-specific differences have not been investigated in relation to COMP levels.

2.8. Limitations in the Literature:

While there has been a great deal of investigation into COMP expression and its' association with OA, there are a number of aspects to the protein that have not been thoroughly investigated. In particular, there is a considerable lack of knowledge regarding the relationship between COMP and PTOA, a distinct sub-type of OA. Furthermore, there is a paucity of research regarding COMP values in populations under the age of 40, over 14 years older than the oldest subjects within this experiment. The pathology of COMP activity at the protein level remains poorly understood, particularly in regards to the driving factors of temporal changes in sCOMP levels in response to physical activity.

Little is known about the fragments of COMP present within blood serum. While the size of serum-based COMP fragments has been established to be primarily between 50-90kDa, the specific pathways that may be associated with each fragment size has only begun to be
investigated. To date, fragments associated with ADAMTS-7, TNF-alpha, and MMP-13 have been definitively identified. However, given that none of these proteins are exclusively associated with OA, or any subtype of OA, the potential prognostic value of specific COMP fragments is not known. Furthermore, there are potentially hundreds of similar inflammatory pathways interacting within not only the knee, but all synovial joints in the human body. Because of the sheer breadth of fragment sizes produced endogenously, it is unlikely that a definitive connection can be made between a specific fragment size and a particular physiological pathway at play.

In regards to sCOMP’s response to physical activity, little has been standardized in terms of total physical exertion. Many studies use self-paced walking for an interval of time, or simply use a total distance of walking to quantify exertion. Both methods, however, will vary based on participant size, speed, and very often in older cohorts, significant mobility issues associated with OA. Furthermore, many of the studies presented have small sample sizes (n=7 to 41 pairs), and therefore a considerable risk of type 2 error. As such, there is no clear evidence to formulate an ideal or preferred quantity of physical activity upon.

There has been very little prospective research evaluating COMP outcomes. Point estimates have been created by many researchers, but these are primarily in older populations (aged 45 and up), with and without the presence of OA. Very few studies have examined sCOMP levels over time. Thus, the potential for associations between sCOMP levels and the progression and development of OA has not been elucidated to date. The relationship between changes in sCOMP levels over time and OA development has not been a prospective research goal in any study to our
knowledge. In the same vein, longitudinal studies investigating COMP expression over time are virtually nonexistent. A great deal of COMP’s behavior over time remains unknown.

Very little is known about COMP behavior in young healthy individuals. COMP assessment is usually performed on older, OA populations and healthy comparison controls. 3,57,58,60,61
3. METHODS

3.1. Study Design:

This is a historical cohort study.

3.2. Ethics Approval

Written informed consent was obtained for each study participants (see appendix I). For participants under the age of 19, the written consent of a legal guardian was obtained. Prior to testing, participants completed a Physical Activity Readiness Questionnaire (appendix II). This was done to ensure the Leger test could be safely completed by participants. Participants were informed that they could revoke their consent at any time, and were under no obligation to complete any portion of the testing protocol. There was no compensation to participants for their participation in the study. Testing was approved by the University of Calgary Conjoint Health Research Ethics Board (CHREB, Ethics ID # E-25075).

Information regarding participants’ overall knee health and the results of their dual x-ray absorptiometry (DXA) body scans were available to the participants after the study. Information believed to be of significant or urgent importance to participants’ health, such as evidence of injury or disease, was communicated to the participant immediately.
3.3. Participant Recruitment:

Two hundred youth/young adults were to be recruited between 2013 and 2015. This included 100 participants who had sustained a sport-related intra-articular knee injury 3-10 years previously in their youth (aged 18 years or younger) and 100 uninjured age, sex and sport matched controls. Sports played included baseball, soccer, basketball, volleyball, skiing/snowboarding, football, horseriding/rodeo, field hockey, rugby, gymnastics, and figure skating. The 3-10 year window post-injury was chosen to examine changes in participant physiology and knee condition, with the assumption that all acute responses to the injury had subsided. Participants were between the ages of 16 and 26 years at the time of recruitment. Age, sex and sport matched participants were universally within +/- six months of age to the injured participant and primarily participated in the same sport at the time of the injured match participant’s injury.

Participants were recruited from one of three sources; previous cohort studies conducted by the Sport Injury Prevention Research Centre (SIPRC), University of Calgary that examined injury risk factors in various youth sports (both injured and uninjured participants), the University of Calgary Sport Medicine Centre (SMC) database (injured participants only) or through personal distribution of study information by study co-investigators, collaborators and participants (uninjured controls only). The injured cohort includes individuals who had sustained an intra-articular knee injury during a previous cohort study, or who had presented to the SMC with a sport-related intra-articular knee injury 3-10 years prior to their date of study participation. In contrast, the uninjured group included individuals who played the same sport, but reported no history of intra-articular knee injury at any point in their lives.
Potential participants were contacted by telephone by a research coordinator who administered a screening interview aimed at determining their eligibility. If eligible, individuals were sent an email containing further information about the study, a consent form, and a Physical Activity Readiness questionnaire (PAR-Q, Appendix II) and information regarding testing at the University of Calgary. Upon completion of these questionnaires, participants were booked for a 3 ½ -hour testing session at the University of Calgary.

3.4. Inclusion/Exclusion Criteria:

For the purposes of this study, intra-articular knee injury was defined as a clinical diagnosis of knee ligament, and/or meniscal and/or other intra-articular, tibio-femoral, or patella-femoral injury (e.g., osteophytes, tibial plateau fractures etc.) that necessitated both a consultation with a medical professional (e.g., physician, physiotherapist) and resulted in disruption of sport participation immediately post injury in the past 3-10 years. Exclusion criteria included pregnancy, non-steroidal anti-inflammatory use or cortisone injection within three months prior to testing, a musculoskeletal injury within 3 months prior to testing that resulted in time loss (work, school or sport), diagnosis of other arthritides, or any current medical problem that prevents participation in the functional testing aspect of the study (e.g., neurological). Medical histories for participants with no previous injury were not required for study inclusion.

Participant height and weight were recorded for the study. Participants were assessed in gym attire with their shoes removed.
3.5. Participant Characteristics: Previous Intra-articular Knee Injury

Injured participants recruited to participate in the study were asked to provide any medical records of treatment and rehabilitation. Qualifying injuries had complete medical records and occurred 3-10 years previously during a sporting activity. Participants who sustained unilateral or bilateral knee injuries, qualified for study inclusion. The knee selected for the study, in the case of bilateral injury, was based on the study physiotherapist’s selection of knee injury with the most complete medical records. The selected knee (hereon referred to as the study knee) was the same knee used for each participant’s matched pair participant (e.g. left vs right knee).

3.6. Test Day Requirements:

Participants were instructed not to eat or drink heavily prior to test sessions, not to have caffeine or cigarettes in the two hours prior to testing, and to avoid any physical activity on test day. No restrictions were placed on participant activity prior to the day of testing. Participants were instructed to bring running shoes and gym attire for the physical activity components of the study.

3.7. Testing Procedures:

Blood samples (4-5ml) were collected from each participant by a certified phlebotomist using standard venipuncture in untreated 5-mL vacutainers. Collections were performed at the very beginning of the test protocol, and again approximately 45 minutes after completion of physical
activity components. As this data collection was included within a larger study protocol, a
number of protocol elements are not directly related to this study.\textsuperscript{86, 87, 88}

Participatn Testing Session Protocol:

Test protocols began at varying times of day, ranging from 9 A.M. to 6 P.M. The test
protocol shown does not reflect the time of day at which testing began. Rather, hour 0:00
indicates the start of the test protocol, regardless of scheduled time of day.

**Hours 0:00-0:15** Consent forms were signed, baseline questionnaires collected, and
PAR-Q forms were completed and reviewed.\textsuperscript{88} Upon completion of consent forms, one 5-
mL vial of blood was collected from the antecubital fossa of every participant’s arm. A
single research coordinator handled all incoming paperwork. This coordinator was
always familiar with the injury history of incoming participants. In order to preserve a
blinding of injury history for study personnel administering strength, balance, and knee
evaluations, they were asked not to discuss injury history with the participant. Blinding
was not always feasible, as many participants had surgical scarring on their knee.

**Hours 0:15-0:20** Anthropomorphic measures (height, weight and waist circumference)
were collected.

**Hours 0:20-2:10** Participants rotated through three stations approximately 35 minutes in
length each for a total of 110 minutes;

**Station 1: Motion Analysis**\textsuperscript{86}:

1. 12-inch drop box vertical jump test (11 total jumps)
2. Five consecutive single leg squats (3 sets/leg, both legs)
3. Single leg stand (70 consecutive seconds, 4 sets/leg)
Station 2: Strength and Balance:

1. Maximal hip adductor exertion test (4 repetitions/leg, both legs)
2. Maximal hip abductor exertion test (4 repetitions/leg, both legs)
3. Maximal hamstring exertion test (4 repetitions/leg, both legs)
4. Maximal quadriceps exertion test (4 repetitions/leg, both legs)
5. Triple Single Leg Hop (3 repetitions/leg, both legs)
6. Star Excursion Balance (3 repetitions/leg, both legs)
7. Unipedal Dynamic Balance (minimum 3 repetitions/leg, both legs, test continues until a minimum score of 2.0 seconds has been achieved on 3 repetitions on each leg).

Station 3: Clinical Knee Exam and Muscle Ultrasound Imaging

The clinical exam was based on a modification of the International Knee Documentation Committee (IKDC) subjective knee examination form and includes active and passive range of motion testing of the tibio-femoral and patella-femoral joints, as well as varus, valgus, anterior and posterior stress tests of the tibio-femoral joint and medial and lateral stress testing of the patella-femoral joint (no physical exertion).

Hours 2:10-2:30 – Participant cleared for Leger test by Certified Exercise Physiologist (CEP) using a PAR-Q, pulse, and blood pressure measurements. Leger test was administered.

Hours 2:30-3:00 – DXA Scanning, remaining questionnaires completed.
**Hours 3:00-3:15** – Collection of post-exercise blood sample, review of questionnaires, and study is completed.

Serum samples were fractionated from whole blood after completion of the day’s testing by centrifugation of samples at 1300G’s for 10 minutes. Serum was extracted from the fractionated blood, and stored at -80°C prior to analysis. Blood was allowed to clot a minimum of 45 minutes with a maximum of 6 hours between collection and centrifugation.

### 3.8. Outcome Methodology

Outcome methodologies describe the protocols used to produce the outcomes of interest in this study and their subsequent analyses. Study analyses are predicated on an effective measurement of the sCOMP fragments in participants’ blood, effective collection of FMI through dual x-ray absorptiometry (DXA), and the administration of the self-reported KOOS symptoms sub-scale score.

#### 3.8.1. Serum COMP (sCOMP) Expression:

The WIESLAB hCOMP assay\(^{18}\) (human Cartilage Oligomeric Matrix Protein quantitative ELISA) was used to quantify the concentration of sCOMP proteins. This assay does not distinguish between intact and fragmented COMP, and binds both with equal affinity.
The ELISA functions using a set of six samples in duplicate of known concentration to produce a reference of absorbance values with which to compare the study samples. A second order regression is performed based on the final absorbance readings of the known concentrations. The resulting equation was used to quantify the unknown concentration of COMP in test samples.

Serum samples were assigned a simple ID# and an A or B to represent pre-exercise or post-exercise serum samples. As all the identifying information had been removed from the samples, the samples were effectively blinded from the researchers for the purposes of this assay. Complete instructions for performing the ELISA are in appendix III.

3.8.2. Fat Mass Index (FMI)

To assess adiposity, a Dual X-ray Absorptiometry (DXA) scanner was employed. DXA scans represent one of the highest standards of evaluation for human evaluation of lean muscle mass, fat mass, and bone mineral density.\textsuperscript{19} While other methods, such as CT scans and MRI’s have ostensibly greater precision, the DXA scanner was the most viable way to assess adiposity at the study’s disposal. DXA scanners are capable of evaluating fat distribution in individual limbs, head, torso, and composite whole-body fat distribution. DXA scanners have been verified as accurate in both adult and adolescent studies, and were therefore a suitable measure for use in this study.\textsuperscript{19} The DXA scanner deployed in the study (Hologic “Discovery A” model, Hologic QDR software ver. 12.6.2.) was calibrated prior to each test session.
DXA scans were performed at the same point in the test protocol for each participant. Participants were asked to lie on their back with their toes pointed inward. Tape was applied to keep participants’ feet together, as the position is difficult to maintain. If the participants’ height exceeded the limitations of the machine (188cm), priority was given to coverage of the feet, and scans of the crown of the head were excluded by necessity.

All participants in the study were scanned, and evaluations of their adiposity were subsequently calculated. Of particular interest were their composite scores, from which all adiposity measures were generated. Composite scores were generated using a combination of a software-provided algorithm and the distribution of landmarks by a single experienced grader (Clodagh Toomey) to mark important distinctions, such as the pelvis, spine, and the boundary points between torso, arms, and legs.

DXA scans use a 3-component method of analysis, classifying tissue as either body fat mass (BFM), fat free mass (FFM), or bone mineral content (BMC). Three-component systems, such as DXA, are restricted by assumptions of the hydration levels of fat free mass (FFM). 4-C models can account for these variations in hydration levels, but were not available for this study. Fat free mass is assumed to have a constant hydration value, but in truth this can be highly variable. Further, tissues situated directly behind bony structures, such as the spine and pelvis, cannot be directly viewed by the x-ray beam. An algorithm must be employed to estimate the relative amounts of adipose and lean tissues present in such locations. Four-component methods are available, however use of such methods require multiple measurements systems, and are prohibitively expensive for most studies. One study evaluating the difference between DXA and
a 4-C model found a systemic underestimation of body fat % in leaner individuals, and a systemic overestimation of body fat % in more obese individuals. Previous studies estimating adiposity by DXA ranged from -5.3% to +2.9% body fat away from the true value.\textsuperscript{76}

Despite these shortcomings, DXA scans have become a standard assessment tool, and is the default tool of many large, multicenter studies such as the National Health and Nutrition Examination Survey, and the Health, Aging, and Body Composition Study. While it cannot be claimed that it is a perfect assessment, or of equal accuracy to a 4-C system, it is the best available tool, and widely accepted clinically. DXA scans have also demonstrated remarkable internal consistency, reporting body fat % coefficients of variation between 0.6% and 1.2%.\textsuperscript{19}

Using dual x-ray absorptiometry, in conjunction with anthropomorphic measures of height and mass, fat mass index (FMI) could be assessed. Of the adiposity assessments available, FMI was selected as the best measure of adiposity available for the purposes of this study.\textsuperscript{19} FMI is a measure of total adipose tissue mass normalized exclusively to the participants’ height. Thus, the total mass or proportion of lean tissue on a participants’ frame does not affect this measure. The elimination of lean mass from adiposity assessments produced a result tied exclusively to total fat mass, which was preferable for assessing research questions linked to adiposity, and not the total weight of the participants.

3.8.3. Knee Injury and Osteoarthritis Outcome Symptom Sub-scale Score:
The KOOS symptom sub-scale score is one of five sections of a self-reported measure designed to evaluate knee related symptoms and function in young active patients with knee injury and OA. The full KOOS questionnaire has been validated in different populations varying in age, disease duration and activity levels, including in young populations (18-46 years) in individuals post-ACL reconstruction, knee arthroscopy and with a history of meniscectomy (with and without OA). Further, it has been shown to have high test-retest reliability (intra-class correlations ranging from 0.78 to 0.97). The KOOS consists of 42 items in five subscales: pain, other symptoms, function in daily living, function in sport and recreation, and knee-related quality of life.

Developed by Roos et al, the KOOS is designed to examine short and long term changes in knee health. The questionnaire typically takes ten minutes to complete, and offers a great deal of insight into the knee condition experienced by the participant. The symptom score is a sub-scale on the KOOS questionnaire pertaining to functional aspects of knee health, including pain, stiffness, catching, and the ability to fully straighten or bend the knee comfortably. The complete set of KOOS symptoms questions is detailed in appendix IV.

3.9. **Primary Outcome: hCOMP ELISA Results:**

Based on the protocol results, a standard curve was produced, and the results were assessed by interpolation of the standard curve. In some cases aberrant absorption readings were produced in the concentration gradient. Due to the linear nature of the absorption values in standard curve,
aberrant readings were apparent when contrasted with the rest of the standard curve values. The clearly aberrant values were dropped when calculating the standard curve formula.

A certain degree of variation was expected within the duplicate results, based on the inherent variability of the reagents and reactions being employed. For all samples tested, duplicate sample values with a coefficient of variation greater than 10% were automatically retested for that participant. The full

3.10. Exploratory Outcome: Fat mass index

Fat mass index (FMI) is a measure of adiposity that is normalized exclusively to participant height. This allows us to measure the adiposity of the participant, without having to adjust for a variable amount of lean tissue mass. Thus, a body builder and a gymnast of equal height and total fat content will have the same FMI, regardless of a dramatic difference in lean tissue mass. Furthermore, variables such as participant sex and age have no effect on the analysis. By adjusting the fat mass index to height (Kg/m²), it allows comparisons between any set of bodies, but makes no distinction on the amount of lean tissue mass present. In this study, the total amount of adiposity is the primary concern.

There are no reference values published for what constitutes “overweight”, “obese”, or any sort of classification based on FMI. Thus, unlike body mass index (BMI) and body fat %, study participants were not dichotomized into traditional classifications, such as “obese”,
“overweight”, or “normal weight”. At this point in time, there is no consensus regarding FMI definitive cut-points identified in the literature. FMI was used to assess any potential linear relationships between adiposity, pre-exercise sCOMP, and ΔsCOMP (change in COMP after physical activity). Exploratory analyses also dichotomized groups based on sex and injury history into high and low adiposity groups based on quartiles. The top 25% of FMI values in each group was considered the “high adiposity” group, while the remaining 75% of each group was considered low adiposity. While some high adiposity group participants may not be considered overweight or obese based on other adiposity measures, these groups are not an indicator of obesity so much as relative magnitude of adipose tissue.

3.11. Exploratory Outcome: KOOS Symptom Score (normalized)

Five symptom based questions were answered on an ordinal scale (e.g., never/rarely/sometimes/often/always). The data was normalized to be represented out of a possible 100 points, the maximum score indicating a healthy asymptomatic knee. The KOOS symptoms sub-scale score was used to assess any potential linear relationships between knee symptoms, pre-exercise sCOMP, and ΔsCOMP values. The full questionnaire is included in appendix IV.

3.12. Exploratory Outcome: Western Blot Results:
Western blot images were assessed using strictly qualitative methods. Based on the visualized staining, the approximate detection limits for maximum and minimum protein molecular weights could be determined with a low degree of precision based on comparisons with an established protein ladder included with each blot. Fragments of sCOMP were tagged with an anti-COMP antibody included in the hCOMP ELISA reagents (Wieslab), and migrated across a gradient of molecular weight. Based on the fluorescence of the bound sCOMP proteins, information regarding the fragment sizes present in each serum sample, as well as the detection limit of the hCOMP kit could be determined. Blotting was also performed to assess qualitative differences between sexes, history of intra-articular knee injury, and total sCOMP concentration. Comparisons of sCOMP based on quantity compared highest quartile (top 25%) and lowest quartile (bottom 25%). Comparisons of injury history and sex exclusively used participants in the middle 50% of sCOMP concentrations as determined by ELISA. A complete account of the western blot protocol is included in appendix V.

3.13. Analyses:

STATA (v13.1, Collage Station, Texas, USA) was used for all statistical analyses. Data was tested for normality (Shapiro-Wilk Test, alpha=0.05) to ensure appropriateness of paired t-test analyses. Scatterplots assessing linear correlations were performed with an intent of producing linear regressions if valid. Covariates (e.g., age, sex, weight, height, BMI, and time since knee injury) at historical cohort study recruitment were summarized using means and 95% confidence intervals.
3.13.1. Assessing Normality

Prior to primary statistical analyses being produced, assessments of normality were generated in order to meet statistical assumptions of parametric analyses (e.g., paired t-tests). Assessments of normality were performed for the within-pair differences of each matched pair of participants in the study using the Shapiro-Wilks assessment of normality. This test is a null-hypothesis analysis comparing the distribution of study data with a normal data distribution. For the purposes of this study, a p-value < 0.05 indicates a non-normal distribution. Normality assessments are included in appendix IV.

3.13.2. Statistical Analyses:

COMP outcomes derived from ELISA testing were subject to results verification prior to acceptance into the final results. Serum samples were tested in duplicate with a requirement that the coefficient of variation between duplicate results may not exceed 10%. In the event of variations exceeding 10%, the values were thrown out, and the sample retested. Intra-plate variability was determined from duplicate results of participant serum only after excising acceptably variant duplicate results. A complete guide to ELISA interpretation is in appendix VI.

Repeated testing resulted in samples experiencing multiple freeze-thaw cycles before a final result could be produced. In the interest of quantifying the potential degradation of samples of
multiple freeze-thaw cycles, one set of samples was subjected to six rounds testing, with a freeze-thaw cycle between each test. Inter-plate variability was established using two control samples provided in each ELISA to determine the variability of results between individual ELISA’s experienced under laboratory conditions.

Once the assumption of an approximately normal distribution of within-pair differences were satisfied, and to account for the matched design, paired t-tests (mean difference; 95%CI) for all primary outcomes were calculated. Primary analyses compared baseline sCOMP levels (pre-exercise), and ΔsCOMP levels between matched pairs using paired t-tests. Analyses were stratified by sex.

Exploratory outcomes were assessed for potential associations between baseline sCOMP, FMI, and KOOS symptom scores. Exploratory assessments were also made between ΔsCOMP, FMI, and KOOS symptom scores. Scatterplots were created to assess potential associations between these outcomes. If these scatterplots did not violate the assumptions for a linear regression (such as evidence of a linear association between variables), a linear regression was performed.

Exploratory assessments of FMI and COMP outcomes were performed based on a dichotomization. Adiposity was compared to pre-exercise and ΔsCOMP using fat mass index (FMI) based on a dichotomization principle. The top 25% of each group was labelled the “high FMI group”, and was compared to the lower 75% in each grouping. Participant groups were divided by injury history and sex. For these tests, there was a risk of non-normal distribution of data. Fat Mass Index data was assessed for normality by a Shapiro-Wilks assessment. There was
evidence of non-normal distributions in both sexes. Consequently, these results were evaluated using non-parametric Mann-Whitney U test, a nonparametric rank-sum test.

Other exploratory outcomes, such as the KOOS symptoms score, were not evaluated using this dichotomization, due to the discreet outcomes produced by the questionnaire. Dichotomization of these results produced outcomes with n-values too low to be statistically reliable.
4. RESULTS

In total, 200 study participants completed baseline data collection for aspects of the study. Of the 200 participants, 192 participants had pre-exercise blood samples collected successfully and 185 participants had both pre-exercise and post-exercise blood samples collected successfully. Missing blood samples were related to difficulties in collecting the samples, (e.g. phlebotomist error during collection, participants withdrawing consent, substantial lower extremity injury in the month prior to participation, and the inability to complete the entire protocol).

4.1. Differences within Successful vs Unsuccessful Blood Collections:

Exclusions occurred when participants did not consent to blood collection, samples could not be collected accurately (phlebotomist error), or the participant had sustained or aggravated a significant injury in the month prior to the test session. Participants who had sustained a recent injury could not complete all aspects of testing protocol, such as jumping, squatting, balance, and Leger tests. Furthermore, acute changes in response to such injuries may affect baseline sCOMP values. Therefore, these participants were excluded from the analysis. However, these participants remained in the study for other aspects, such as KOOS scores, physical activity participation, dietary information, and other study elements remained of great utility to the greater study aims.
Table 4.1. Differences within Successful vs Unsuccessful Blood Collection Participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collected n=85</td>
<td>Not Collected n=5</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.80 (1.78, 1.82)</td>
<td>1.82 (1.78, 1.86)</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>80.3 (78.2, 82.4)</td>
<td>94.6 (76.7, 112.4)</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>24.8 (24.2, 25.4)</td>
<td>28.6 (22.8, 34.4)</td>
</tr>
<tr>
<td>FMI (Kg/m²)</td>
<td>4.1 (3.8, 4.4)</td>
<td>5.7 (2.4, 9.1)</td>
</tr>
</tbody>
</table>

Due to these restrictions, a total of 85 matched pairs were included in the primary analysis (90 females and 80 males). These 170 participants were also used for exploratory analyses. Participants whose matched pair was disqualified from the analysis were also excluded from all analyses.

4.2. Participant Characteristics:

Injured participants presented with a variety of different intra-articular knee injury types. These injuries were categorized into several subcategories to more fully describe the injuries involved in the study. These types included exclusive meniscal tears, tears of the anterior cruciate ligament (ACL) and/or posterior cruciate ligament (PCL), injuries to other ligaments, bony fractures (e.g., tibial plateau), and catchall other category (e.g., knee subluxations, dislocations,
and combination ligament/meniscal injuries). Due to the inclusive criteria for knee injuries, a diversity of injury types were identified

<table>
<thead>
<tr>
<th>Injury Type</th>
<th>Meniscal Injury</th>
<th>Full ACL/PCL Tear</th>
<th>1-3° MCL/LCL Sprain or 1-2° ACL/PCL sprain</th>
<th>Bone Fracture (Tibial Plateau)</th>
<th>Other (Patellor-femoral Dislocation or Subluxation,)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>8</td>
<td>14</td>
<td>7</td>
<td>1</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>30</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td>45</td>
</tr>
</tbody>
</table>

PCL; Posterior Cruciate Ligament, MCL; Medial Cruciate Ligament, LCL; Lateral Collateral Ligament.

The proportions of each type vary somewhat by sex, the most notable difference being the large proportion of full ACL/PCL tears present in the female population (30/45, or 66.7%) compared with male participants (14/40, or 35%).

Some participants who have experienced an intra-articular knee injury have experienced injuries in both knees (study knee & other knee). In total, 8/40 injured male participants (20%) had experienced bilateral knee injuries. In total, 10/45 injured female participants (22.2%) had experienced bilateral knee injuries.
Study participants were matched on age, sex, and sport during the recruitment process. Anthropomorphologic baseline characteristics, including relevant data on time since intra-articular injury of the study knee was reported.

| Table 4.3. Participant Characteristics |
|---------------------------------------|---|---|---|---|
| Characteristic                        | Male | Female | Male | Female |
| Time Since Injury                     | Injured n=40 | Uninjured n=40 | Injured n=45 | Uninjured n=45 |
| (Months) Median, Range                | 79.8 (35.1, 118.5) | NA | 85.5 (44.3, 124.2) | NA |
| Age (Years) Median, Range             | 19 (17, 26) | 19 (18, 26) | 23 (16, 26) | 23 (15, 26) |
| Height (m) Mean, 95% CI               | 1.80 (1.77, 1.82) | 1.80 (1.77, 1.82) | 1.67 (1.65, 1.69) | 1.67 (1.65, 1.69) |
| Weight (Kg) Mean, 95% CI              | 83.1 (79.9, 86.3) | 77.4 (74.5, 80.2) | 68.0 (64.6, 71.5) | 65.1 (62.0, 68.2) |

Each injured participant’s age, sex, and sport-matched control differed in age by no more than six months. Age and height did not differ between study groups. Previously injured participants had a higher weight than uninjured. For the female injured cohort, the mean time of study entry was 86.2 months (7.18 years) since time of injury, compared to males which was 78.7 months (6.6 years). The time since injury does not appear to be statistically different between sexes.
Injured participants played a variety of youth sports at the time of intra-articular knee injury. Participants were recruited into the study for intra-articular knee injuries that occurred during these youth sports. Uninjured controls were matched on age, sex and sport-specific sport participation at the same age. The distribution of sport types is summarized below.

<table>
<thead>
<tr>
<th>Sport Played</th>
<th>Males (n=40 matched pairs)</th>
<th>Females (n=45 matched pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseball</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Basketball</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Dance</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Field Hockey</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Football</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Figure Skating</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Hockey</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>Horseback Riding</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Rugby</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Skiing/Snowboarding</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Soccer</td>
<td>6</td>
<td>21</td>
</tr>
<tr>
<td>Track/Running</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Volleyball</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>
There are some differences between sexes in regard to the sports played amongst participants. Some sports, such as hockey, soccer, and football have notable disparities in representation based on sex.

4.3. **Data Assessments**

Participant outcomes were assessed to confirm a variety of underlying assumptions regarding the data. Issues around the normality of outcome distributions were assessed for primary and exploratory outcomes. In addition, assessments of the hCOMP ELISA’s consistency and variability detecting COMP in serum samples tested over repeated freeze-thaw cycles were performed. Finally, potential linear relationships were assessed between sCOMP outcomes and outcomes of particular interest, including adiposity, KOOS symptom scores, and time elapsed between exercise completion and post-exercise blood serum collection.

4.4. **Primary Results: COMP Levels & Matched Pair Comparisons**

The primary outcomes of hCOMP levels in study participants were derived by the hCOMP ELISA kit (Wieslab). Outcomes are presented by pre-exercise, post-exercise, and change after exercise by sex and injury group. Differences between injury groups were assessed by a paired t-test. The 95% confidence interval and p-value associated with each test is presented in the table.
Table 4.5. Primary Results: Serum COMP Levels & Matched Pair Comparisons

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Male (n=40 per group)</th>
<th>Female (n=45 per group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Previous</td>
<td>No Previous</td>
</tr>
<tr>
<td></td>
<td>Intra-articular Knee Injury</td>
<td>No Previous Injury</td>
</tr>
<tr>
<td>Pre-Exercise COMP ng/ml (mean), 95% CI</td>
<td>1685.1 (1470.7, 1899.4)</td>
<td>1465.6 (1275.2, 1656.0)</td>
</tr>
<tr>
<td>Pre-Exercise COMP (median, range)</td>
<td>1699.2 (577.5 - 3189.6)</td>
<td>1442.8 (537.3 - 2612.0)</td>
</tr>
<tr>
<td>Post-Exercise COMP ng/ml (mean), 95% CI</td>
<td>1701.0 (1479.9, 1922.1)</td>
<td>1577.7 (1396.4, 1758.9)</td>
</tr>
<tr>
<td>Post-Exercise COMP (median, range)</td>
<td>1735.8 (522.0, 3183.7)</td>
<td>1552.0 (532.0, 2818.5)</td>
</tr>
<tr>
<td>Delta COMP ng/ml (mean), 95% CI</td>
<td>15.9 (-117.8, 149.7)</td>
<td>112.1 (47.2, 271.4)</td>
</tr>
<tr>
<td>Delta COMP (median, range)</td>
<td>19.76 (-1015.16, 802.21)</td>
<td>101.45 (-1267.13, 1173.81)</td>
</tr>
</tbody>
</table>

*Significant findings (p<0.05) are **bolded** within the table.

The primary results of the study identified some differences within sCOMP baseline by history...
of intra-articular knee injury and sex. In male participants, baseline COMP values were substantially higher than female participants in both injured and uninjured cohorts. Males with a history of intra-articular knee injuries demonstrated a significantly elevated baseline sCOMP level than male participants without a history of intra-articular knee injury. In female participants, there was no evidence of a significant difference in baseline sCOMP values between groups with and without a history of intra-articular knee injury.

Delta COMP outcomes were positive within each group, indicating a slight elevation of sCOMP values in response to physical activity. In both male and female participants, there was no difference in the magnitude of the Delta COMP value between groups. Injured females appeared to elevate more sharply than uninjured females, but the difference in Delta COMP were not significant between groups. In the male participants, an opposing trend was seen: uninjured males appeared to have a greater Delta COMP value than injured males. However, these results were also not statistically significant.

Statistical evaluations were not performed for differences in post-exercise sCOMP outcomes, as they were a combination of the effects of baseline sCOMP values and Delta sCOMP values. As these are two distinctly unique phenomena of sCOMP characteristics, no meaningful assertions could be made regarding the cause of any differences identified between study groups.

4.5. Exploratory Participant Outcomes

Exploratory participant outcomes assessed outcomes not related the the primary hypotheses and analyses. Measurements of adiposity measures and knee-related symptoms as reported by the
KOOS were reported.

<table>
<thead>
<tr>
<th>Table 4.6. Exploratory Participant Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
</tr>
<tr>
<td>Weight; (Kg) mean, 95% CI</td>
</tr>
<tr>
<td>FMI (kg/m$^2$) mean, 95% CI</td>
</tr>
<tr>
<td>KOOS Symptom Score (normalized) mean, 95% CI</td>
</tr>
</tbody>
</table>

*Significant at $p < 0.05$; BMI; body mass index, FMI; fat mass index, KOOS; knee osteoarthritis outcome score

Previously injured males and females demonstrate a substantial elevation of overall adiposity in comparison with uninjured counterparts. In both sexes, previously injured participants exhibited a greater presence of knee symptoms based on the KOOS symptom scores. (e.g., a lower value on the KOOS symptoms score). Lohmander et al (2003) reports a difference of 8 points in any KOOS outcome as being a clinically significant difference. The magnitude of difference between KOOS symptom scores approximates the threshold for clinical relevance between injured and uninjured groups in study participants.

Participants demonstrated similar differences in other KOOS subscales. However, as these were not part of the planned analyses, they have been excluded from the results.
4.5.1. Exploratory Results: Fat mass index vs Pre-Exercise COMP

Exploratory assessments were made to investigate the possibility of a linear relationship between FMI and baseline sCOMP values. Simple scatterplots were created to assess the potential relationship. Scatterplots were divided by sex and history of intra-articular knee injury.

![Fat Mass Index vs Pre-Exercise COMP, Male Cases](image1)

![Fat Mass Index vs Pre-Exercise COMP, Female Cases](image2)

**Figure 4.1. Fat mass index vs Pre-Exercise COMP, Previously Injured Participants**

Scatterplots compare fat mass index and baseline sCOMP in participants with no history of intra-articular knee injury. Scatterplots divided by sex.

Scatterplots compare fat mass index and baseline sCOMP in participants with a history of intra-articular knee injury. Scatterplots divided by sex.

Figure 4.1 shows no evidence of a linear relationship between FMI and pre-exercise COMP values. This was the case in both male and female participants with a history of intra-articular knee injury.
Figure 4.2. Fat mass index vs Pre-Exercise COMP, No Previous Injuries

Scatterplots compare fat mass index and baseline sCOMP in participants with no history of intra-articular knee injury. Scatterplots divided by sex.

Figure 4.2 shows no evidence of any relationship between FMI and pre-exercise COMP outcomes. As with the previously injured participant, neither males nor females appeared to show any meaningful relationship between the primary outcome and adiposity.

Given the tremendous amount of variance within both FMI and pre-exercise COMP values, further examination of these data included dichotomizing each subgroup into high and low FMI values and comparing participants’ pre-exercise COMP values. Dichotomization into highest 25%ile and lowest 75% was chosen as there is no established cut-off value for overweight or obesity on the FMI scale. Analyses were performed by sex and injury group using a Mann-Whitney U test (a non-parametric version of the student’s t-test).
Table 4.7. Pre-Exercise sCOMP Outcomes: Previously Injured Participants
Dichotomized by FMI

<table>
<thead>
<tr>
<th></th>
<th>Observations</th>
<th>Median Pre-Exercise sCOMP (ng/ml)</th>
<th>z-score</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High FMI (&gt;5.55 kg/m²)</td>
<td>10</td>
<td>1642.0</td>
<td>0.312</td>
<td>0.75</td>
</tr>
<tr>
<td>Low FMI (&lt;5.55 kg/m²)</td>
<td>30</td>
<td>1785.2</td>
<td>0.312</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High FMI (&gt;7.61 kg/m²)</td>
<td>12</td>
<td>987.7</td>
<td>0.257</td>
<td>0.80</td>
</tr>
<tr>
<td>Low FMI (&lt;7.61 kg/m²)</td>
<td>33</td>
<td>1128.3</td>
<td>0.257</td>
<td></td>
</tr>
</tbody>
</table>

Significant at p < 0.05 FMI; fat mass index, High FMI; top quartile of participants, Low FMI; all other participants.

Analyses based on dichotomization of fat mass indexes found no evidence of a difference in pre-exercise sCOMP outcomes in relation to fat mass index values in both males and females without a history of intra-articular knee injuries.
Table 4.8. Pre-Exercise sCOMP Outcomes: Participants With no Previous Injury Dichotomized by FMI

<table>
<thead>
<tr>
<th></th>
<th>Observations</th>
<th>Median Pre-Exercise sCOMP (ng/ml)</th>
<th>z-score</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High FMI (&gt;4.31 kg/m²)</td>
<td>10</td>
<td>1270.9</td>
<td>0.375</td>
<td>0.71</td>
</tr>
<tr>
<td>Low FMI (&lt;4.31 kg/m²)</td>
<td>30</td>
<td>1551.6</td>
<td>0.375</td>
<td></td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High FMI (&gt;6.84 kg/m²)</td>
<td>12</td>
<td>1144.8</td>
<td>-0.231</td>
<td>0.82</td>
</tr>
<tr>
<td>Low FMI (&lt;6.84 kg/m²)</td>
<td>33</td>
<td>1116.9</td>
<td>-0.231</td>
<td></td>
</tr>
</tbody>
</table>

*Results significant at p < 0.05 FMI; fat mass index, High FMI; top quartile of participants, Low FMI; all other participants.

Analyses based on dichotomization of Fat Mass Indexes found no evidence of a difference in pre-exercise sCOMP outcomes in relation to fat mass index values in both males and females without a history of intra-articular knee injuries.

4.5.2. Exploratory Results: Fat mass index vs ΔsCOMP

Exploratory descriptive analyses examining the associations between ΔsCOMP and FMI were based on scatterplots examining potential linear associations between ΔsCOMP and fat mass index.
Figures 4.3 and 4.4 demonstrated no evidence of any linear association between $\Delta s\text{COMP}$ and FMI. However, an additional analysis was performed to confirm this apparent lack of association.

To further investigate the potential for association between FMI and $\Delta s\text{COMP}$, groups were next dichotomized into high and low FMI categories based on the 25th percentile. A non-parametric analysis was performed comparing the COMP outcomes of the participants in the top 25% of

**Figure 4.3. Fat Mass Index vs $\Delta s\text{COMP}$, Previously Injured Participants**
 Scatterplots compare fat mass index and $\Delta s\text{COMP}$ in participants with a history of intra-articular knee injury. Scatterplots divided by sex.

**Figure 4.4. Fat Mass Index vs $\Delta s\text{COMP}$, No Previous Injury**
 Scatterplots compare fat mass index and $\Delta s\text{COMP}$ in participants with no history of intra-articular knee injury. Scatterplots divided by sex.
adiposity with the remaining 75%. A Mann-Whitney U-test was performed (a non-parametric t-test), as the data has been shown not to be normally distributed within the study groups. This test operates on a null hypothesis that there is no difference between groups, producing a p-value representing the probability that such a distribution would be produced given the null hypothesis is true.

Table 4.9. Delta sCOMP Outcomes: Previously Injured Participants Dichotomized by FMI

<table>
<thead>
<tr>
<th></th>
<th>Observations</th>
<th>Median Delta sCOMP (ng/ml)</th>
<th>z-score</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>High FMI (&gt; kg/m² 5.55)</td>
<td>10</td>
<td>139.6</td>
<td>-1.187</td>
</tr>
<tr>
<td></td>
<td>Low FMI (&lt; 5.55 kg/m²)</td>
<td>30</td>
<td>-47.7</td>
<td>-1.187</td>
</tr>
<tr>
<td>Female</td>
<td>High FMI (&gt;7.61 kg/m²)</td>
<td>12</td>
<td>45.3</td>
<td>0.103</td>
</tr>
<tr>
<td></td>
<td>Low FMI (&lt;7.61 kg/m²)</td>
<td>33</td>
<td>89.9</td>
<td>0.103</td>
</tr>
</tbody>
</table>

Significant at p < 0.05 FMI; fat mass index, High FMI; top quartile of participants, Low FMI; all other participants

Analyses based on dichotomization of Fat Mass Indexes demonstrated no evidence of a difference in delta sCOMP outcomes in relation to fat mass index ratings in both males and females with a history of intra-articular knee injuries.
### Table 4.10. Delta sCOMP Outcomes: Participants With no Previous Injury Dichotomized by FMI

<table>
<thead>
<tr>
<th></th>
<th>Observations</th>
<th>Median Delta sCOMP (ng/ml)</th>
<th>z-score</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>High FMI (&gt; 4.31 kg/m²)</td>
<td>10</td>
<td>-46.6</td>
<td>0.875</td>
</tr>
<tr>
<td></td>
<td>Low FMI (&lt; 4.31 kg/m²)</td>
<td>30</td>
<td>103.4</td>
<td>0.875</td>
</tr>
<tr>
<td>Female</td>
<td>High FMI (&gt; 6.84 kg/m²)</td>
<td>12</td>
<td>-107.4</td>
<td>1.386</td>
</tr>
<tr>
<td></td>
<td>Low FMI (&lt; 6.84 kg/m²)</td>
<td>33</td>
<td>93.8</td>
<td>1.386</td>
</tr>
</tbody>
</table>

Significant at p < 0.05 FMI; fat mass index, High FMI; top quartile of participants, Low FMI; all other participants

Analyses based on dichotomization of Fat Mass Indexes revealed no evidence of a difference in ΔsCOMP outcomes in relation to fat mass index values in both males and females without a history of intra-articular knee injuries.

#### 4.5.3. Exploratory Results: KOOS Symptoms Scores (Normalized) vs Pre-Exercise COMP

Exploratory hypothesis stated a positive association between increasing KOOS symptoms and sCOMP values. Furthermore, exploratory hypotheses stated a potential association between increasing KOOS symptoms and an increasing magnitude of ΔsCOMP. Scatterplots were created to assess the potential associations between COMP outcomes and the KOOS symptom scores.
Figure 4.5. KOOS Symptoms vs Pre-Exercise COMP, Previously Injured Participants
Scatterplots compare KOOS symptoms and baseline sCOMP in participants with a history of intra-articular knee injury. Scatterplots divided by sex.

Figure 4.6. KOOS Symptoms vs Pre-Exercise COMP, No Previous Injuries
Scatterplots compare KOOS symptoms and baseline sCOMP in participants with no history of intra-articular knee injury. Scatterplots divided by sex.

Scatterplots in figures 4.5 and 4.6 do not show any evidence of an association between pre-exercise COMP in cases or controls (male or female). Further assessment of a potential relationship between KOOS symptoms and sCOMP outcomes was limited, due to the ordinal nature of the KOOS symptoms outcomes and small sample sizes.

4.5.4. Exploratory Results: KOOS Symptoms Score (Normalized) vs ΔsCOMP
Exploratory hypotheses regarding KOOS symptoms and FMI were based on associations between delta COMP and the outcome of interest. Therefore, scatterplots were created to assess the potential associations between ΔsCOMP and the covariates of interest. Additionally, KOOS symptoms and FMI were dichotomized into high and low outcomes comparing ΔsCOMP outcomes. The first such assessment is based on the self-reported KOOS symptom score and ΔsCOMP.

Figure 4.7. KOOS Symptoms vs ΔsCOMP, Previously Injured Participants
Scatterplots compare KOOS Symptoms and ΔsCOMP in participants with a history of intra-articular knee injury. Scatterplots divided by sex.

Figure 4.8. KOOS Symptoms vs ΔsCOMP, No Previous Injury
Scatterplots compare KOOS Symptoms and ΔsCOMP in participants with no history of intra-articular knee injury. Scatterplots divided by sex.

There was no evidence to support a linear association between KOOS symptoms and ΔsCOMP in any group presented on figures 4.7 and 4.8.
4.6. Western Blotting

The first western blot was performed on samples of COMP protein provided as part of the ELISA hCOMP kits (Wieslab) using an anti-COMP antibody provided. The purposes of this blot were two-fold; the first goal was to test the range of fragment sizes of the COMP fragment provided in the hCOMP kit. The second goal was to test the molecular weight detection limits of the anti-COMP antibody within the ELISA kit.

4.6.1. COMP Fragments from ELISA Detection Kit

This western blot demonstrated the range of detectable COMP fragments being provided from the COMP ELISA assay. Due to the varying concentrations being tested, only the lanes marked 1 and 2 were clearly identifiable. When compared to the protein ladder of established molecular weights (left side of image), a range of detectable COMP fragments was determined.
Figure 4.9. ELISA Protein Verification

Protein Ladder (left side) indicates the locations of specific molecular weights (kDa) within the gel. Arrow indicators (1 & 2) define duplicate wells of differing concentrations of human COMP sourced from the ELISA kit. Image was contrasted to maximize visibility of experimental lanes.

Pre-exercise samples were selected to provide a wide range of samples based on injury status, participant sex, and COMP concentrations as determined by ELISA. Priority was given to investigating COMP values that were the most extreme in concentration. Therefore, samples in this blot were selected based on sCOMP concentrations in the top and bottom quartiles among study participants. As in the previous blot, each sample was tested in duplicate, as represented on the image by the labelled arrows.

4.6.2. Initial Blotting (All Participant Types)
### Table 4.11. Participant Characteristics for Western Blot in Figure 4.10

<table>
<thead>
<tr>
<th>Well Location as indicated by arrows. [Performed in Duplicate Wells]</th>
<th>Injury Status</th>
<th>Sex</th>
<th>COMP Values (low=&lt;1500ng/mL) (high = &gt;3000ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uninjured</td>
<td>Male</td>
<td>Low</td>
</tr>
<tr>
<td>2</td>
<td>Uninjured</td>
<td>Female</td>
<td>Low</td>
</tr>
<tr>
<td>3</td>
<td>Injured</td>
<td>Male</td>
<td>Low</td>
</tr>
<tr>
<td>4</td>
<td>Uninjured</td>
<td>Female</td>
<td>High</td>
</tr>
<tr>
<td>5</td>
<td>Injured</td>
<td>Male</td>
<td>High</td>
</tr>
<tr>
<td>6</td>
<td>Injured</td>
<td>Male</td>
<td>High</td>
</tr>
<tr>
<td>7</td>
<td>Injured</td>
<td>Male</td>
<td>Low</td>
</tr>
</tbody>
</table>
Figure 4.10. Initial Blotting (All Participant Types)
Duplicate wells are indicated by the arrows. The protein ladder (far left of image) indicates the range over which COMP was detectable in each well. The bromophenol blue line indicated on the lower image is an artifact of the blotting procedure, and is not relevant to the blot. Image was contrasted to maximize visibility of experimental lanes.
Figure 4.10 showed no evidence of a qualitative difference in sCOMP fragmentation patterns between sexes in both low and high concentration COMP serum. Furthermore, no differences were identified between high and low COMP values within either sex.

Given the low amount of total samples tested, more blotting was warranted. Further assessments were targeted specifically to participants with major intra-articular knee injuries and to evaluate potential differences based on sex. COMP levels were approximately equal across participants.

4.6.3. Participants with Previous Injury

This Western Blot was performed to investigate any potential sex-based differences present within participants who had experienced a traumatic intra-articular knee injury. Of particular interest were sex-based differences between participants. Study participants had sCOMP levels in the middle 50% of participants based on injury group and sex (e.g. between 25th and 75th percentiles).
Table 4.12. Participants with Previous Intra-articular Knee Injury for Western Blot in Figure 4.11

<table>
<thead>
<tr>
<th>Lanes (tested in duplicate)</th>
<th>ID#</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>97 Pre-Ex</td>
<td>Female</td>
</tr>
<tr>
<td>2</td>
<td>87 Post-Ex</td>
<td>Male</td>
</tr>
<tr>
<td>3</td>
<td>87 Pre-Ex</td>
<td>Male</td>
</tr>
<tr>
<td>4</td>
<td>58 Post-Ex</td>
<td>Female</td>
</tr>
<tr>
<td>5</td>
<td>58 Pre-Ex</td>
<td>Female</td>
</tr>
<tr>
<td>6</td>
<td>45 Post-Ex</td>
<td>Male</td>
</tr>
<tr>
<td>7</td>
<td>45 Pre-Ex</td>
<td>Male</td>
</tr>
</tbody>
</table>
Figure 4.11. Participants with Previous Injury

Duplicate wells are indicated by the arrows. The protein ladder (far left on image) indicates the range over which COMP was detectable in each well. The blotches at the top of duplicates 2-4 are artifacts from the experiment. The image was contrasted to maximize the visibility of experimental lanes.

The Western Blot showed no evidence of a difference in fragmentation patterns between male and female participants who had experienced a previous intra-articular knee injury. Despite the presence of artifacts disturbing parts of duplicates 2-4, the rest of the blot was notably clear of artifacts. There is inconclusive evidence of fragment detection below 30kDa, but it does not appear consistently across all samples.

4.6.4. Participants with No Previous Injury
The next Western Blot was performed to investigate any potential sex-based differences present within participants who did not experience a traumatic intra-articular knee injury. Of particular interest were sex-based differences between participants. Participants included in this blot possessed approximately average sCOMP concentrations (between 25\textsuperscript{th} and 75\textsuperscript{th} percentile within the study group, (uninjured, dichotomized by sex).

<table>
<thead>
<tr>
<th>Well Location as indicated by arrows.</th>
<th>ID#</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Performed in Duplicate Wells]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>88 Pre-Ex</td>
<td>Male</td>
</tr>
<tr>
<td>2</td>
<td>77 Post-Ex</td>
<td>Male</td>
</tr>
<tr>
<td>3</td>
<td>77 Pre-Ex</td>
<td>Male</td>
</tr>
<tr>
<td>4</td>
<td>53 Post-Ex</td>
<td>Female</td>
</tr>
<tr>
<td>5</td>
<td>53 Pre-Ex</td>
<td>Female</td>
</tr>
<tr>
<td>6</td>
<td>44 Post-Ex</td>
<td>Male</td>
</tr>
<tr>
<td>7</td>
<td>44 Pre-Ex</td>
<td>Male</td>
</tr>
</tbody>
</table>

The 4\textsuperscript{th} Western Blot compared male and female participants with no history of intra-articular knee injury. Brackets four and five distinguish female serum samples from male serum samples.
Figure 4.12. Participants with No Previous Injury

Each arrow bracket indicates a pair of duplicate wells. Numbers on the left indicate known molecular weights from the protein ladder. The contrast has been manipulated to maximize the visibility of protein staining in each lane. The lack of fluorescence in lane 5 is an artifact, rather than a sample lacking in COMP expression.

A clear comparison can be made between bracket 4 (female) and brackets 1-3 (male). However, lanes 5-7 have experienced an excessive amount of scrubbing during the final blot processing, and cannot be qualitatively evaluated. The western blot found no evidence of differences in the fragmentation pattern of sCOMP between uninjured males and females within the study.

4.7. Summary of Results

Primary analyses demonstrated a statistically significant elevation in baseline sCOMP values amongst male participants with a previous intra-articular knee injury when compared with
uninjured matched controls. Previously injured males demonstrated an average sCOMP value 15% higher than uninjured controls. In female participants, no statistically significant difference in baseline sCOMP values was found. Baseline values of sCOMP were substantially higher in males than females regardless of injury history. Previously injured males experienced mean baseline sCOMP values 41% greater than previously injured females. Males with no previous injury experienced mean baseline sCOMP values 24% higher than females with no previous injury history.

Mean ΔsCOMP values were found to be positive across all study groups. No differences were found in the magnitude of ΔsCOMP values within matched pairs in males or females. There is no evidence to suggest the magnitude of ΔsCOMP values differed by sex.

Exploratory analyses assessed the potential for linear relationships between primary outcomes of sCOMP and ΔsCOMP values to adiposity as measured by FMI, and to knee symptoms as reported by the KOOS symptom score. Exploratory scatterplots showed no evidence of a relationship, linear or otherwise, between the primary outcomes and variables of interest. Dichotomized outcomes of FMI also found no evidence of a relationship between adiposity and sCOMP, or adiposity and ΔsCOMP outcomes.

Western blots assessed the detection limits of sCOMP values produced for primary analyses. Fragments were detected ranging from full length proteins of 535-kDa to a minimum of approximately 30-kDa. While it cannot be said whether all fragments within this range were detected, it is certain that no fragments below the minimum of 30-kDa were detected during
ELISA tests for the primary sCOMP outcomes. Blots were used to assess qualitative differences in fragmentation patterns between sexes, injury history, and relative abundance of sCOMP present within serum samples. No qualitative differences were found between any of these groups.
5. DISCUSSION

This research is the first study of its kind. Within sCOMP research, this is the first study to employ a matched historical cohort design to evaluate baseline levels of sCOMP, and the potential contributors to ΔsCOMP values in response to physical activity. While sCOMP studies have been performed in adolescent\textsuperscript{10} and elderly\textsuperscript{21} populations, this study fills a relatively unexplored age range (16-26 years) in regards to sCOMP research.

This research found a statistically significant increase in baseline sCOMP values amongst previously injured male participants compared to matched controls. No difference was found between previously injured female participants and their matched controls. Participants having experienced a previous intra-articular knee injury tended to have greater adiposity measures and experienced greater amounts of knee dysfunction as measured by the KOOS symptoms score. Exploratory analyses to assess potential associations between fat mass index (FMI) and KOOS symptoms to sCOMP and ΔsCOMP outcomes yielded no statistically significant associations. Such findings did not preclude the possibility of such associations existing, however the evidence shown here does not support the assertion of any such associations.

5.1 Participant Characteristics:

Within the matched-pairs, there are some clinically relevant differences in baseline characteristics. Males with no previous intra-articular knee injuries weighed less and had a higher body mass index and fat mass index than their matched-pair uninjured counterparts.
Previously injured female participants demonstrated substantially higher values across weight, BMI, and fat mass index in comparison with uninjured controls. In both sexes, participants with a previous intra-articular knee injury possessed higher body mass index and fat mass index than their uninjured matched-pairs.

Participants with a previous intra-articular knee injury were experiencing significantly worse symptoms than their uninjured counterparts. The magnitude of mean differences in KOOS scores (7.9 in males, 8.4 in females) represents a clinically significant difference in knee dysfunction between study groups of both sexes. This suggests that even at this early time point, participants have already begun to experience differences in knee symptoms related to a previous intra-articular knee injury. In both knee function and adiposity, previously injured participants are substantially different than their uninjured counterparts. The associations of these differences and sCOMP outcomes are addressed in the exploratory analyses.

5.2. Primary Results: Pre-Exercise COMP Levels & Matched Pair Comparisons

The primary findings of the study are based on the average matched-pair differences (mean, 95%CI) in sCOMP levels prior to physical activity. Analyses were dichotomized by sex as the literature suggests that sex-specific differences are likely present within the study. There were no statistically significant differences in female mean sCOMP outcomes. Estimated means were within 1% between study groups.
Previously injured males demonstrated significantly greater baseline sCOMP values in comparison with their uninjured matched controls. The mean matched pair difference between injured and uninjured males in the study was 219.5 ng/ml (95% CI 11.0, 428.0). Previously injured males expressed mean sCOMP values an average of 15% (95% CI 1.0%, 33.6%) higher than their matched-pair. Currently, no research has suggested a minimum clinically significant difference in baseline sCOMP values in regards to prognosis or diagnosis of any form of OA.

That said, studies have correlated osteoarthritic progression measured on the Kellgren-Lawrence scale in knees with an increase in baseline sCOMP values in one and three year cohorts aged forty and higher.\(^2\),\(^7\) The present study has only a single measurement point to evaluate. Given that male participants were rigorously matched, it can be interpreted that the injured male cohort has experienced an increase in baseline sCOMP values since the intra-articular injury occurred. It is probable that this increase correlates with worsening osteoarthritic conditions within the affected knee joint.

To date, no studies have investigated sCOMP levels in relation to intra-articular knee injuries.\(^1\) However, there are many potential explanations for the sex-specific differences in baseline sCOMP between participants with and without a history of intra-articular knee injury. A likely contributor is the disparity in injury types between sexes. Females experienced a much higher proportion of ACL injuries than male participants, which could be a contributing factor. In female participants, 30/45 (66.7%) of study qualifying injuries were complete ACL tears. In male participants, only 14/40 (35%) participants were complete ACL tears. The difference in physiological response to the type of injury likely has an effect on sCOMP baseline values. Alternately, it is possible that the physiological response to an intra-articular knee injury is
fundamentally different in regards to sCOMP levels between sexes. Sex differences have been reported in other literature; however these sex-specific differences were identified in participants with a minimum age of forty five, substantially higher than the ages in this study. Another possibility lies in the speed and intensity of returning to sport after injury. Sex-specific differences in both time off of sport, and the intensity of sport returned to could both contribute to the sex-specific differences in baseline sCOMP. For example, sCOMP levels in runners were observed to have elevated levels of sCOMP immediately prior to a marathon in comparison with healthy controls. The authors suggested that this elevation was related to increased articular matrix turnover as a consequence of such intense training. A pilot study of college aged competitive female soccer players found that sCOMP levels increased over the course of a season, and that increases in sCOMP were qualitatively associated with increased playing time. This suggests that at both moderate and high intensity of sport there is positive association between baseline sCOMP and total physical activity.

Age-related differences in sCOMP values have been noted in both sexes when compared to other major sCOMP studies. Males aged 45 to 65+ with a Kellgren-Lawrence grade of 0-1 (healthy joints), of both Caucasian and African-American descent (n=151) had median sCOMP values increase with age from 743.3 to 1031.8 ng/ml. In males aged 45 to 65+ with a K-L grade of 2-4 (substantially degraded joints), of both Caucasian and African-American descent (n=170), median sCOMP values increased with age from 790.4 to 1066.5 ng/ml. Similar trends can be observed when comparing the median sCOMP values in female participants; females aged 45 to 65+ (n=151) with a K-L grade of 0-1 had median sCOMP values increase with age from 631.9 to 1031.8 ng/ml. Females aged 45 to 65+ (n=297) with a K-L grade of 2-4 had median sCOMP
values increase with age from 744.1 to 1075.4 ng/ml.\textsuperscript{73} Within the Johnston OA trials, there is statistically significant evidence of an increase of log transformed sCOMP values (a mathematical transformation of the data) with age in participants aged forty five and up, independent of sex, ethnicity, or presence of radiographic OA ($r^2=0.10$, $p=0.0001$).

Younger cohorts have also demonstrated age related differences in sCOMP. One study of 82 healthy controls aged 22 and under found that controls under 16 years had significantly higher baseline sCOMP values than participants aged 16 and older.\textsuperscript{13} This clear change in sCOMP expression with age suggests that skeletal maturity is a significant contributor to sCOMP levels in younger populations. Taken with studies in older cohorts, it appears that sCOMP values are relatively high during skeletal immaturity, and decline after maturity completes. Decades later, the trend reverses, and age-related degradation within the body serve to increase sCOMP levels regardless of the presence or absence of OA.

When comparing the median values between the Johnston OA study and the present study, median values in all groups were substantially greater than in the older cohorts at all age ranges.\textsuperscript{73} It would appear that turnover rates drop intrinsically between the age of this cohort (16-26 years) before reversing at or before the age of the Johnston OA cohort (45+ years). Differences in baseline sCOMP among younger cohorts above or below 16 years suggest that sCOMP values in this study had likely dropped from the years prior.

It is possible that systemic hormonal differences are contributing to the substantial differences in sCOMP values between this cohort and those of the Johnston OA trial. Changes in hormones
such as testosterone, progesterone, estrogen, and testosterone may have substantial effects on both osteoarthritic development and sCOMP outcomes.\textsuperscript{54} To date, we are not aware of any research investigating the link between sCOMP and hormonal activities. Changes in hormones, in particular changes due to menopause, are likely contributors to the differences in median sCOMP levels seen between different age groups.

5.3. ΔsCOMP Levels and Matched Pair Comparisons

ΔsCOMP levels (Post-exercise sCOMP – Pre-exercise sCOMP) were assessed for every participant who completed the entire protocol. As predicted in the literature, mean ΔsCOMP values were positive (elevated) in both sexes in injured and uninjured groups. There was no significant difference in the magnitude of ΔsCOMP between groups in either sex. In fact, injury history did not appear to have a consistent effect on the magnitude of ΔsCOMP among participants. Previously injured females presented no differences in mean ΔsCOMP values than uninjured females (mean paired difference = 116.50 ng/ml, 95% CI -79.08, 312.08). In male participants, the results found no evidence of a difference in ΔsCOMP amongst uninjured participants (mean paired difference = -96.1 ng/ml, (-303.1, 110.8). Therefore, in both sexes there is no evidence of a significant difference in paired ΔsCOMP values.

Other studies investigating ΔsCOMP support the conclusion that OA status does not affect ΔsCOMP outcomes.\textsuperscript{53} In a study of medial knee compartment OA, no differences in ΔsCOMP outcomes was found between participants with healthy or afflicted knee joints. While this study involved developed, diagnosed arthritis in participants aged 40-74, these findings are in
agreement with the present study.

The magnitude of elevation varied greatly in individuals, ranging from tremendous increases to substantial decreases. These changes in ΔsCOMP were of comparable magnitude to those in the literature,\(^21\) which ranged from decreases of up to 35% to increases of over 60% from baseline. While COMP levels were thought to elevate to a minor degree with some consistency, the findings of this study do not support that conclusion.

The large range of ΔsCOMP values appears to have multiple contributing factors. The mean within-plate coefficient of variance between duplicate ELISA samples initially averaged 9.8%, suggesting that some fluctuation of ΔsCOMP was a limitation of the ELISA. Each sample was tested in duplicate, it is likely contributor to the apparent variation in proportional changes in ΔsCOMP values.

It is also possible that COMP fragments are being degraded to below the detection limit of the ELISA kit. Precipitous drops in COMP could be attributed to a large proportion of the sCOMP falling below this detection limit, although blots within the literature have consistently found a similar detection limit ranging from 25 to 30 kDa.\(^{67,79}\) It is also possible that some participants degrade COMP in response to exercise in such a way that it is not detected by the sCOMP antibody. There is no evidence to suggest that such degradation would be categorically different between groups, however, and is unlikely to have a meaningful effect on quantitative ELISA outcomes.
5.3.1. ΔsCOMP vs KOOS Symptoms Score (Normalized)

Assessments for the association between ΔsCOMP and KOOS symptoms were evaluated, within sex and injury status. The scatterplots demonstrated no evidence of any association between KOOS symptoms and ΔsCOMP in any group. To date, we are not aware of any literature that has assessed the potential association of these two values. Currently, there is no reason to postulate any association between these two outcomes.

5.3.2. ΔsCOMP vs Fat mass index w/Regression & 95% CI

Assessments for ΔsCOMP and FMI association were evaluated within each sex and injury status. In each assessment, scatterplots were created plotting the two variables. Scatterplots yielded no evidence of any associations between ΔsCOMP and fat mass index. Further dichotomized assessments also showed no evidence of any association between ΔsCOMP and FMI. Based on the results of the scatterplots and Mann-Whitney U-test, it was determined that there is no evidence that adiposity plays any role in the magnitude of ΔsCOMP values in males or females with or without a history of intra-articular knee injuries. While not an explicit outcome of interest in previous research, other studies investigating changes in sCOMP after physical activity have not identified any associations between adiposity and ΔsCOMP.21,45

5.3.3. Pre-Exercise COMP vs KOOS Symptoms Scores
There was no evidence of any association between KOOS symptom scores and ΔsCOMP values in men or women regardless of injury history. To date, we are not aware of any literature that has attempted to compare KOOS symptoms and sCOMP values. This exploratory analysis provides no evidence to suggest any association between the two KOOS symptoms and baseline sCOMP values.

5.3.4. Pre-Exercise COMP vs Fat mass index (FMI)

Scatterplots assessed as subgroups of injured and uninjured participants grouped by sex showed no evidence of any associations between baseline sCOMP and FMI outcomes. No clear trends were discernible by injury status or sex, and there were no unifying features within either sex or injury group. There is no significant evidence of an association between fat mass index values and pre-exercise COMP values. This finding is at odds with the findings of the Johnston County OA project, which found an association between body mass index (BMI) and baseline ln sCOMP values. The lack of any clear association within the present study, despite contrary evidence, is likely due to a combination of substantially lower n-values (n=769 vs n=45), simpler statistical methods, and a categorically different range of participant ages. The lack of a significant association between baseline sCOMP and FMI in this study does not prove a lack of association in general.

Nonparametric dichotomized analyses also yielded no significant evidence to support an association between baseline sCOMP and FMI. Therefore, it was concluded that the analyses show no evidence to suggest an association between pre-ex COMP and FMI. Despite the lack of
evidence, this association may yet exist. However, a greater n-value and more advanced analyses may be required to uncover the association, should it exist.

5.4. Western Blots

Western blots were performed to investigate both the nature of the COMP fragments detected by the ELISA kit, as well as to validate the primary results produced by the study. Previous studies have used the Wieslab ELISA kit with a similar age range (mean age = 27 years, 95% CI +/- 3.8) to produce mean sCOMP values similar to those produced in this study.\(^8\) Other sCOMP assays have produced minimum fragment detection limits of approximately 25 to 30 kDa.\(^1\) However, the array of fragment sizes has not been assessed for this ELISA.

In addition, western blots were used to investigate potential differences in sCOMP fragmentation patterns among different sCOMP expression levels, sex, and history of previous intra-articular knee injury.

5.4.1. Figure 4.9: COMP Fragments from ELISA Detection Kit

The purpose of the figure 4.9 was a proof of concept that COMP could be visualized using the antibodies and human COMP samples provided in the ELISA (Wieslab). The western blot demonstrated Wieslab provided COMP was consistently detectable above a minimum of 30kDa. It was unclear if the COMP tested in this blot existed below 30kDa, or if this was the detection limit of the COMP antibody, under which the COMP fragments were too degraded to be
detected. It is not known if all fragments above 30kDa were detected. However, it is clear that degradation of sCOMP below 30kDa makes the protein undetectable within the Western blots, and therefore fragments of under 30kDa would most likely also be undetectable within the sCOMP ELISA used to produce primary results.

5.4.2. Figure 4.10: Initial Blotting (All Participant Types)

Figure 4.10 sought to identify the detection limits of the COMP antibody by using study participant sCOMP samples. The detection limits of sCOMP fragments appeared identical, or nearly so, to blots performed using kit-provided human COMP. Therefore, the detection limit of 30kDa was determined to be a function of the antibody’s ability to bind to fragmented COMP, and it could be hypothesized that the antigen recognition sites in COMP that are specific to this antibody are completely degraded in fragments below 30kDa, rather than a categorical difference in the COMP being tested. This is in agreement with other sCOMP recording methods, which also have difficulty binding COMP fragments below this threshold.59,65,81

Previous studies have demonstrated that COMP may have distinct fragmentation patterns depending on the health status of the participant. While advanced OA is not distinguishable from healthy controls, a categorical difference in fragmentation was noted between participants with rheumatoid arthritis and other study participants.10 Based on this evidence, these blots sought to investigate differences in COMP’s fragmentation pattern and spread by sex, injury status, and sCOMP concentration, as determined by the top and bottom quartiles within study groups. None
of the samples tested showed a distinctive pattern compared to other samples. Other studies have demonstrated distinct fragmentation patterns that correlate to specific degradation pathways, including ADAMTS-7, TNF-alpha, and MMP-13 (catabolites present in cartilage). However, unlike the methodology used to investigate specific COMP epitopes, the polyclonal antibody employed in this study detected a substantially wider range of fragments, preventing the isolation of specific fragment sizes within the blot.

Within the blots, some lanes fluoresced a great deal more clearly than others. However, there was no evidence to suggest a qualitative difference in the fragmentation pattern between individual participants on the basis of sex, injury status, or abundance of sCOMP. A more precise method of fragment capture appears necessary to successfully distinguish between specific sCOMP epitopes.

5.4.3. Figure 4.11: Participants with Previous Injury

Figure 4.11 examined only participants with a previous intra-articular knee injury. Both sexes were represented, with samples collected at baseline and post exercise. No differences were found in the distribution of COMP fragmentation between time points within the participants evaluated. Fragmentation appeared much the same as in previous blots, with no clear delineation between sexes.

During testing, the magnitude of change in sCOMP between baseline and post-exercise samples was not considered. It is possible that participants with particularly large ΔsCOMP values may
have produced differing fragmentation patterns. Furthermore, it is possible that specific injuries, such as meniscal damage or specific ligament damage may have produced distinct differences in sCOMP distribution between time points. To date, blots investigating specific injury types and the magnitude of ΔsCOMP after physical activity have not been performed.

5.4.4. Figure 4.12: Participants with No Previous Injury

The blots in figure 4.12 examined participants without a previous intra-articular knee injury. As with the third blot, the purpose was to investigate sex differences, and changes in sCOMP fragmentation pre to post exercise. As with previous blots, there was no evidence of a differential fragmentation pattern among sCOMP in any sample tested. The lack of differences in sCOMP fragmentation patterns based on sex or history of previous intra-articular knee injury is in agreement with previous studies in COMP fragmentation.65

5.5. Limitations:

There were some inherent limitations in the research methodology. Participants were recruited from the Sport Injury Prevention Research Centre study cohorts, Sport Medicine Centre at the University of Calgary, and through solicitations within the department of Kinesiology. These participants may be more highly educated, may be generally more physically active and may have less adipose tissue than the general population. The requirement of a history in youth sport also biased the recruitment to more physically active individuals. Recruitment of previously injured participants from the Sport Medicine Centre implied a certain level of care and
rehabilitation undertaken after the intra-articular knee injury of interest, which may have been well above the typical care received in the general population. These inherent differences suggest that the study population, both with and without previous injuries, differ in meaningful ways from the general population within this age range. It can be noted however, that given the matched study design that uninjured participants are subject to similar potential differences and thus generalizability may be limited rather than inherent selection bias.

Participants in this study’s age range have varying degrees of skeletal maturity. Skeletal growth typically ceases near age 14 in females, and 15 in males. Although peak bone density, a key measure of skeletal maturity, may not be achieved until as late as 30 years, more than 90% of peak bone density occurs by age 19 in females, and age 20 in males. Study participants >19 (91.1%) for females and >20 (71.3%) for males are expected to be near full skeletal maturity, whereas the remaining participants are anticipated to be potentially skeletally immature. While no association was found between age and sCOMP outcomes, this may be related to a small sample size and potential type II error. One study found that healthy participants aged 12 to 16 years showed higher mean baseline sCOMP values than healthy participants aged 16 to 22. It is likely that our study’s age range is a few years past where detectable differences in sCOMP due to age could be identified.

The timing of testing sessions was of concern initially. sCOMP values are known to fluctuate during the day, and it was possible that the variance between session times could contribute to fluctuations in COMP significantly. Some studies suggest that COMP levels are lowest during nighttime rest, but quickly recover to a consistent baseline following breakfast and early morning
activity.\textsuperscript{10} It is possible, however, that COMP levels remained low for early morning participants. A minority of participants arrive for sessions as early as 9 a.m. however these participants were instructed to eat breakfast prior to testing, and should have sCOMP baselines at or very near average daily baselines.

The sCOMP levels present in each participant were derived from serum samples. Blood serum relies on the distribution of loose and degraded sCOMP from fluid in all synovial joints to derive a serum concentration for each individual participant. Collecting COMP values from serum is of substantially less utility than COMP values derived from synovial fluid. Serum samples approximate cartilage turnover for the entire body, not just the joints of interest. Synovial fluid COMP levels are much more responsive to the conditions of the specific joint being sampled. Serum samples were chosen over synovial fluid as the cost of synovial fluid collection is substantial, and blood serum was required for other study outcomes.

Given the transient nature in COMP changes post exercise, there is some concern over the precise amount of time allowed from completion of the exercise portion to the second blood draw. To compensate, we have calibrated the study to allow approximately 45 minutes from completion of exercise to the second blood collection. In practice, while the average time is very close to 45 minutes, there is a large degree of variability between individual participants. In the literature, COMP appears most elevated immediately after physical activity for a limited period of time. The 45 minutes between completion of activities and post-exercise blood collection may eliminate much of the COMP elevation the study intends to capture.\textsuperscript{5} In addition to potentially failing to capture the transient elevation, the differing elapsed times between physical challenge
and post-exercise blood collection may confound the results somewhat. Analysis of the results found no evidence of an effect on delta COMP values based on this variable.

The time that elapsed between collection and storage of serum samples may be variable. Samples were stored in a $-80^\circ$ C freezer between one and six hours after sample collection. During the time between collection and storage, active proteinases within the blood samples continued to degrade proteins including COMP. While it is unlikely that this degradation produced substantial changes in measurable COMP in this period, the differences in time prior to storage is variable between samples, and a source of minor variation within the study.

Serum samples within the study were, on occasion, tested as often as 2-3 times before acceptable results were produced. Each testing involved thawing the samples from $-80^\circ$ C to a liquid phase at approximately $0^\circ$ C. Each freeze-thaw cycle degrades the COMP protein, depressing the total amount detectable by ELISA. However, as demonstrated by the test-retest values over multiple freeze-thaw cycles presented in the results, it is unlikely that any samples used in the analysis were degraded to a significant degree by multiple freeze-thaw cycles.

The changes in COMP after exercise may be associated with the magnitude of exertion given by each participant. For example, the Leger test assesses maximum cardiovascular capabilities in a maximal effort fashion, such that each level completed is incrementally more challenging than the previous level. Thus, participants completing ten levels exert dramatically more effort than a participant who completes three. This disparity may influence the ability to evaluate meaningful differences in the change of COMP expression post-exercise. Compounding this issue is the
reality that not all participants give truly maximal exertion during the test. Unlike other studies examining changes in COMP, this study did not standardize the physical activity performed to the same degree given the complexity and breadth of outcome measures included.\textsuperscript{3,21,60}

Perhaps the greatest source of error in the study is measurement bias. COMP has a half-life of 7.4 hours\textsuperscript{10} and as such some of the plummeting values (-2/3 of total value) over a 3 hour time frame may be explained by poor reliability of $\Delta$sCOMP. The large range in delta COMP outcomes are partially explained by physiological variability in participants, as proportionally large $\Delta$sCOMP values have been reported in the literature\textsuperscript{21}. Finally, there is an unknown proportion of degraded sCOMP that may not be detected by the ELISA. The quantity and characteristics of these undetectable sCOMP fragments are unclear.

The exploratory analyses within this study were negatively affected by non-normal distributions in KOOS scores and adiposity measures and a small sample size. A multivariable linear regression was not possible. Exploratory analyses did not benefit from the matched-pair design present in primary analyses, introducing the potential for confounding factors rising from age and youth sport. Exploratory analyses were limited by relatively low n-values in comparison with primary findings.

While this study has revealed a great deal about COMP, and its progression during pre-radiographic OA, there is a great deal still unknown. The mechanisms of transient changes in COMP are poorly understood, as is the change in COMP immediately following a significant knee injury. The effects of COMP in the years immediately after injury are not known, and
cannot be predicted within this study. This unknown activity could pose a confounding effect upon the results.

There remains a large gap in knowledge regarding the sex-specific responses of articular chondrocytes and how this may relate to COMP. Chondrocytes, synoviocytes, tendons, and ligaments of the knee have all been implicated in producing sex-hormones, and differential expression of hormone receptors and biological pathways. Because of this, care must be taken not to generalize results for the entire study that may only be relevant or true in one sex. This is a difficult issue, as sex-specific differences have seen little study in the literature. As such, analyses are consistently dichotomized by sex in the results.

There remains much unknown about the COMP detected in the WIESLAB hCOMP assay. While most COMP in blood is in 50-90kDa fragments, range of fragment sizes detectable by ELISA was not stated in the kit. To alleviate this issue, Western Blotting was performed on several serum samples from both control and injury groups, as well as on the purified COMP from the ELISA kit to confirm the detectable range of COMP protein. It should be noted that relatively few samples were tested by western blotting (n=20) compared with primary ELISA testing. The lower amount of test results is a limitation on the assessments. However, it was felt that further testing was not justified, as no meaningful differences in COMP fragmentation were detected in any of the initial blots.

This study is focused on a particularly young population and completed within the context of PTOA. As such, any meaningful conclusions formed are relevant only within this young active
subset of the population. On the whole, OA afflicts a much broader range of people and
generalizability beyond the study sample target population should be considered with caution.

5.6. **Strengths**

This study has a number of strengths that make it uniquely suited to provides new insight into
COMP characteristics. The matched-pair design of the study allowed the study to disregard any
potential confounding due to age, youth sport, and sex during primary analyses. To date, this is
the largest study of sCOMP values in response to physical activity, and the first study
investigating COMP in such a young cohort. Furthermore, this is the only study investigating
sCOMP in the wake of a major intra-articular knee injury in the context of post-traumatic OA.
These traits all point to a gap within the literature of COMP, and demonstrates the niche filled by
this research.

This study benefited from being part of a larger multidisciplinary research program. Because of
the many disciplines included on this project, strictly biological features like COMP and changes
in COMP due to exercise could be derived.

5.7. **Future Directions:**

This research has demonstrated sex-specific differences in baseline sCOMP related to history of
intra-articular knee injury. Furthermore, there appears to be a substantial elevation in COMP
values between younger cohorts in comparison with older cohorts. Paradoxically, COMP appears
to increase with age in persons aged forty five years and up. There is an apparent gap in knowledge around study participants in the ages between these cohorts. Future research could bridge these age groups to investigate COMP during this period.

This study did not investigate the potential effects of specific injury type on sCOMP characteristics. While the majority of study participants experienced reconstructed ACL injuries, the type of intra-articular injury may have a chronic effect on COMP values years after the injury. As this research adds participants, performing analyses based on injury type will become far more viable, and should be investigated as the study progresses. As evidenced by the Johnston County OA study,\textsuperscript{45} ethnicity appears to play a significant role in baseline COMP values. The potential interaction between ethnicity and changes in COMP has not been investigated. Future research within this study would do well to collect data on ethnicity, as this could play a substantial role in COMP characteristics, both at baseline and in response to physical activity.

While high COMP levels have been associated with osteoarthritic progression, it is clear that this protein alone is not capable of producing a diagnosis of OA. However, it is possible that COMP evaluations, in conjunction with other biomarkers, could demonstrate real predictive value of incipient or worsening OA. Biomarker analyses including COMP could prove beneficial in all types of OA, not just the post-traumatic variety.

In future investigations of delta COMP outcomes, a more standardized regimen of physical activities must be employed. Changes in COMP may yet prove to have substantial value in
regards to the prediction and assessment of OA. Yet, this will only be possible in the physical activities are tightly controlled in terms of pace and distance. Absent these controls, it is unlikely that such measurements will produce meaningful results.

Other aspects of this study have employed analyses of magnetic resonance imaging (MRI) performed on each participant at baseline. MRI’s produce detailed images of a number of features within the intra-articular space of the knee. In particular, elevated sCOMP values have been associated with osteophyte formation, a characteristic clearly visible on MRI. Other features, such as cartilage thickness and presence of articular legions may also provide insight into sCOMP and post-traumatic OA.

It has been suggested in the literature that specific neoepitopes of COMP can be tied to specific degradative pathways and catabolites within articular cartilage. The ELISA testing for COMP performed within this study does not discriminate between these neoepitopes. Rather, this study measured the value of all COMP fragments present within serum. Future studies may benefit from focusing on specific fragment sizes of COMP, and the relative abundance of fragments in comparison with other neoepitopes, and total sCOMP concentrations. Such comparisons may provide new insights into degradative pathways more closely linked to OA and PTOA than total sCOMP values.
6. CONCLUSIONS

The research has demonstrated sex-specific differences in baseline sCOMP values between participants with and without a traumatic intra-articular knee injury. Previously injured male participants showed a relatively small (15%) increase in sCOMP values compared to their uninjured controls. This significant elevation of sCOMP values indicates greater rates of articular turnover compared to uninjured controls, and is a likely signal of greater amounts of joint degeneration compared to their uninjured controls. Female study participants did not show a similar elevation in baseline sCOMP values. This discrepancy is likely related to differences in injury type between groups, physiological and hormonal differences relating to sex, and psychological differences relating to the rate and intensity of return to sport. At this point it is unclear which of these factors are the most significant.

ΔsCOMP values did not vary in any meaningful way between study groups in either sex. ΔsCOMP may still represent a valuable research avenue. However, limitations in the study of this design crippled the quality of the data. Similar experiments under more stringent guidelines surrounding the timing of sample collection and exercise protocols may produce more meaningful results in the future.

Experimental analyses showed no evidence of association between KOOS symptoms and any sCOMP outcome, both at baseline and in response to physical activity. It is likely that no such association exists. Experimental analyses of fat mass index and sCOMP outcomes also showed no such association. While associations between ΔsCOMP and adiposity have not been
investigated in the literature, baseline sCOMP has been linked to body mass index. Therefore, an association may still be uncovered in the future when more data becomes available.

Future directions for this research should include greater focus on the types and total number of intra-articular knee injuries. The potential relationship between sCOMP and injury types and quantities is largely unexplored. The potential effects of age and adiposity on sCOMP, while not demonstrated in this study, are potential contributors to overall sCOMP values. Future research should continue to investigate age and adiposity, as the effects may not be visible until many years into the study.

In regards to ΔsCOMP values, this research has demonstrated a clear need for greater standardization of physical activities for participants. ΔsCOMP values cannot be meaningfully assessed without a precise protocol that is strictly followed. Changes in COMP related to physical activities may prove to have profound research value, but only if employed correctly within the study. Future research demands this in order to produce research of value.

The success of such future research may also depend on a greater capability to identify specific fragment types and their relative abundance on the whole. Therefore, future research should invest in more specific evaluations of COMP fragmentation sizes, and their relative proportion to total COMP values.

While not explored in this research, magnetic resonance imaging (MRI) exists for the majority of study participants. Some articular features, such as osteophyte formation, have been reportedly
associated with increases in sCOMP. Few studies have the ability to evaluate associations between MRI-visible features and COMP outcomes. This historical cohort study has that capability and future investigations in this regard are planned. Comparisons between sCOMP and information regarding the articular structure of the knee available from MRI’s may produce new insights that have not yet been discovered. Although COMP research is hardly new, research such as this offer unique opportunities to combine bench-level evaluations of COMP with high quality imaging, medical histories, and clinical and performance outcomes. The combination of information from so many disparate fields will likely produce new and unique insights in the immediate future.
References


78. Mateer, J., Hoch, J., Mattacola, C., Butterfield, T., & Lattermann, C.- Serum cartilage oligomeric matrix protein levels in collegiate soccer athletes over the duration of
I. APPENDIX I. PARTICIPANT CONSENT FORM

All participants signed a participant consent form prior to testing. Participants under the age of majority had a legal guardian sign on their behalf.
CONSENT FORM

TITLE: Secondary Prevention of Osteoarthritis Following Joint Injury in Youth Sport: A Mixed Methods Study

FUNDING: International Olympic Committee, CIHR Team OA, CIHR STAIR Team in Injury Prevention in Youth, and CIHR OOG MOP 1335970.

INVESTIGATORS
Dr. Carolyn Emery PhD PT, Dr. Linda Woodhouse PhD PT, Dr. Jackie Whittaker, PhD PT, Allison Ezzat MSc PT, Dr. Carly McKay PhD, Dr. Roman Krawetz PhD, Dr. Reed Ferber PhD, Dr. P. Tish K. Doyle -Baker DPH PhD, Dr. Preston Wiley MD, Dr. Deborah Marshall PhD, Dr. Mariana Brussoni PhD, Dr. Janet Ronsky PhD, Dr. Jacob Jaremko MD PhD, Dr. Raylene Reimer PhD, Dr. Benno Nigg PhD, Dr. Brent Edwards PhD, Dr. Vincent Von Tscharner PhD, and Dr. Gregor Kuntze.

This consent form is only part of the process of informed consent. It should give you the basic idea of what the research is about and what your participation will involve. If you would like more detail about something mentioned here, or information not included here, please ask. Take the time to read this carefully and to understand any accompanying information (as well as to discuss this research project with your child, if applicable). You will receive a copy of this form.

BACKGROUND

Osteoarthritis (OA) is a leading cause of chronic pain and disability. OA currently affects about 4.6 million Canadians and it is expected that this number will double in the next thirty years. The most common joint affected by OA is the knee joint. OA is a complicated disease and is caused by the interaction of multiple risk factors. The two most important risk factors are previous joint injury (often experienced in youth) and obesity. Sport participation is the leading cause of joint injury in adolescents, and knee injuries, which are associated with an increased risk of early OA, are one of the most common. In addition to the early onset of OA, joint injuries are also known to have physical, emotional, and economic impacts.

Once an individual has experienced a knee joint injury it is important that they are monitored and compared to individuals that have not had an injury as they may hold vital clues that can assist in the early detection and development of treatments aimed at slowing the progression of OA. These clues may be revealed by studying: physical activity participation and fitness, chemicals found in blood, the trunk and leg muscles, behaviors attitudes and beliefs related to injury and sport participation and health care utilization (i.e., visits to physicians and physiotherapists). There is a critical need for research that will lead to both joint injury and OA prevention in youth and young adults with a sporting background. You have been selected to participate in this study due either to your history of acute knee joint injury or due to the fact that you have not experienced a knee joint injury. 200 participants are expected to take part in this 3-year study.

WHAT IS THE PURPOSE OF THE STUDY?

The purpose of this project is to determine whether or not youth and young adults with a history of knee injury in the past 3 – 10 years differ in knee joint structure, physical activity participation, aerobic
fitness, adiposity, blood biomarkers, the ability to perform movement tests, trunk and leg muscle size, behaviors, attitudes and beliefs associated with injury and sport participation, nutrition, and health care utilization, compared to youth and young adults with no history of intra-articular knee injury.

**WHAT WOULD I HAVE TO DO?**

If you choose to participate in the study you will undergo a short phone interview (approx. 10-15 minutes) with a research coordinator to determine if you are eligible to participate and if you are willing to consent to participating in the study. Testing will take place once a year for three consecutive years at University of Calgary Sports Medicine Centre and Human Performance Laboratory. Yearly testing will begin with us sending you: the Physical Activity Readiness (PAR-Q), Baseline and Nutrition questionnaires via email. You will be asked to complete these at home and if applicable, with a parent. You will then be scheduled to come into the University of Calgary Sports Medicine Centre for a testing session, which will be approximately 3.5 hours in length. You will be provided with precise directions and appointment times that suit both yourself and the researchers. Upon arrival at the University’s of Sports Medicine Centre, you will be met by a researcher who will provide you with a parking pass and instructions for parking, and will be asked to participate in various testing stations. These will include:

1. **Two blood tests.**
   This is to help identify presence of biomarkers (chemicals) that are commonly found in people with early OA. One blood test will be done prior to exercise, the other after.

2. **Completion of the following questionnaires (as applicable):**
   a. Numerical Pain Rating Scale
   b. Godin Leisure-Time Physical Activity Questionnaire
   c. Knee Injury and Osteoarthritis Outcome Questionnaire
   d. ACL-Quality of Life Questionnaire
   e. Athletic Identity Measurement Scale
   f. Exercise Identity Scale
   g. EuroQol 5 Dimensions Questionnaire
   h. Intermittent and Constant Osteoarthritis Pain questionnaire
   i. The Personal Health Questionnaire - 9

3. **Height, weight, and waist circumference.**

4. **Aerobic fitness test (20m shuttle run)**
   For this test, you’ll be asked to travel 20m distances within a set time. The set time decreases as the test progresses. You would initially be walking to complete the 20m distance, and eventually progress to jogging/running. The test ends when you can no longer complete the distance in the set time.

5. **An ultrasound imaging assessment of your abdominal, back, hip and knee muscles.** An experienced physiotherapist will administer this while you are lying down.

6. **Functional tests including:**
   a. **Triple single hop test**, which involves two trials of hopping on one leg, three times, trying to get as far as possible.
   b. **Star excursion balance test**, which involves balancing on one foot while reaching out as far as possible with the other leg, in three directions.
   c. **Unipedal dynamic balance test** which involves standing on a balance pad with one foot, closing your eyes, and raising your other leg.
   d. **Vertical drop jump**, which involves dropping down from a 31cm platform, and jumping back up as high as possible, ten times. Motion analysis will be used during this test.
   e. **Single leg squat**, which involves squatting with one leg at a time on a force plate for ten repetitions per leg. Motion analysis will be used during this test.
f. **Single leg balance**, which will involve balancing on one leg on a force plate with your eyes open and then with your eyes closed. Ten repetitions will be done on each leg. Motion analysis will be used during this test.

**NOTE:** PLEASE LET US KNOW IF YOU HAVE AN ALLERGY TO ADHESIVES. IF SO, YOU WILL NOT BE ABLE TO COMPLETE FUNCTIONAL TESTS (d) AND (e).

7. **Leg strength testing.** You will be asked to lay sideways on an exam table and be instructed to push your leg up, against a dynamometer (measurement tool) with maximal force, three times for each leg.

8. **A dual-energy X-ray absorptiometry scan (DEXA).** For the scan you will be asked to lay flat on an exam table while the arm of a machine passes over you from head to toe to measure your fat and muscle mass. This test is an x-ray. It will take about 10 minutes and should not give you any discomfort. You will be asked to participate in a physical activity monitoring assessment using an accelerometer device (ActiGraph GT3X). Specifically, you will be asked to wear a small, lightweight device attached via an elastic belt around your waist for a period of 7 days.

9. You will be asked to record your food and fluid intake for a 3-day duration (2 weekdays and 1 weekend day). These do not have to be recorded on consecutive days but should reflect your typical eating patterns. You will be given information on how to accurately complete the diary, via a web-based app called MyFitnessPal.

In addition to the yearly testing sessions, you will be asked **ONCE** over the three years to undergo x-ray and MRI studies of both knees. One of our research coordinators will contact you individually, perform a screening interview (asking questions about pregnancy, metal objects in your body etc.) and provide you with details for booking an imaging appointment at a time that is convenient for you. Both the x-ray and MRI studies will happen at the same time and location. The X-ray studies will take approximately 10 minutes and involve scans taken standing and lying down. The magnetic resonance imaging (MRI) studies, will require you to lie on your back inside an MRI scanner for 20-30 minutes while scans of your knee are performed. You will be holding a button, and if you are uncomfortable for any reason you can push the button to communicate with a technician. If you cannot lie still enough for us to perform a high-quality scan, are uncomfortable or anxious while in the MR scanner, or you want to stop for any reason you can be removed from the scanner immediately. You will not be given a sedative or injected with any intravenous contrast material. If you are pregnant at the time you will not be eligible for the x-ray or MRI studies. Further MRI studies may not be performed if you have certain metal objects inside your body, or are too claustrophobic to enter the scanner.

Additionally, we will be contacting you every 6 months to ask about your healthcare utilization since we were last in contact with you. This will help us to compare healthcare utilization, between individuals who have had a knee injury and those that have not.

Finally, if appropriate, a research coordinator may contact you about other opportunities to participate in related research projects. Your decision to participate in these related projects will not in anyway influence or effect your involvement in this study.

**WHAT ARE THE RISKS?**

There are no expected risks associated with participating in this study. The measurements described above will be done under close supervision and every effort will be made to ensure your safety. As with any physical activity there is the possibility of a muscle pull or strain for the running and jumping tests. The risks of the
muscle function tests include the possibility of muscle injury and soreness. The risk of injury will be reduced by careful supervision during the testing procedures.

The blood tests will be done in a standardized fashion as done in any laboratory that withdraws blood. The person taking your blood will either be a trained technician or a physician. You should let the person taking the blood know if you have any allergies. Although very rarely, there is the possibility of local infection within days of having your blood taken. You would need to see a physician for this and be treated with antibiotics. There is also the remote possibility of fainting, which would be related to having a needle. This is unpredictable. This resolves after lying down for a short period. You would recover completely from this.

The estimated dose of radiation from the DEXA scan is less than 25 mrad, and from knee x-rays, 20 mrad. No amount of radiation is considered to be completely safe. For the sake of comparison, the dose from a chest x-ray is 25 mrad, from a dental x-ray is 750 mrad, natural living at sea level exposes you to 100 mrad and watching TV one hour per day exposes a person to 1 mrad per year. The actual health risks from exposure to low x-ray doses are difficult to determine. Conservatively, health experts assume radiation health risks are proportional to exposure. This leads to pessimistic estimates of a 0.01% chance of developing cancer due to a 10 000 µSv x-ray dose, compared to a normal lifetime risk of cancer for women in the US of 33% (Reference: Kalender WA. Effective dose values in bone mineral measurements by photon absorptiometry and computed tomography. Osteoporosis Int 2: 82-87, 1992). Magnetic resonance (MR) is a technique that uses magnets and radio waves, not radiation, to take pictures of the body. MRI has no known harmful effects as long as you have none of the risk factors, which you will be screened for in the pre-MRI screening interview. Specifically, you should not have an MRI if you have a pacemaker or certain other metal objects inside your body, especially around the eyes, because the strong magnets in the MR scanner might cause these to heat up or move, causing harm. You will also need to remove all metal from your clothing and pockets; otherwise these objects could be pulled into the magnet and cause harm. No metal can be brought into the magnet room at any time, since the magnet is always "on".

There may be some initial discomfort wearing the physical activity monitor and belt. However, the belt is fully adjustable to fit the individual and minimize any discomfort.

**ARE THERE ANY BENEFITS?**

If you agree to participate in this study there may or may not be a direct medical benefit to you. It is unlikely that your risk of developing OA will decrease during the study. The information we get from this study may help us to provide better injury and OA prevention in the future through adolescent programs and sport. However, you will receive information about your body mass index, bone mineral density, % fat and lean body mass, leg balance and strength, aerobic fitness as well as the results of your MRI and x-ray tests.

**DO I HAVE TO PARTICIPATE?**

No, you do not have to participate. Participation is completely voluntary. If you agree to participate, we require you to sign and return this form to us. Two copies of this form have been provided. Please keep one for your records, and return the other to us.

Your signature on this form indicates that you have understood to your satisfaction the information regarding participation in this research project and agree to be a participant. In no way does this waive your legal rights nor release the investigators, sponsors, or involved institutions from their legal and professional responsibilities.

You are free to withdraw from the study at any time by contacting the Research Coordinator at 403-220-3394 or kneestudy@ucalgary.ca. Continued participation should be as informed as your initial consent, so feel free to ask for clarification or new information, throughout your participation in the study. If there is new information available throughout this study period, you will be informed as soon as possible.

**WILL I BE PAID FOR PARTICIPATING, OR DO WE HAVE TO PAY FOR ANYTHING?**
Participants will not be paid to participate in the study, and there will be no costs (parking permits will be provided) to the participants.

**WILL MY RECORDS BE KEPT PRIVATE?**

All of the information collected will remain strictly confidential. Your privacy will be assured. Only the investigators responsible for this study, the research assistants who will be doing the assessments and data analysis, and the University of Calgary Conjoint Health Research Ethics Board will have access to this information. Data will be kept in a secure, either locked, or password protected location, for five years after completion of the study. Confidentiality will be protected by using a study identification number in the database. Any results reported from the study will in no way identify study participants.

**IF I SUFFER A RESEARCH-RELATED INJURY, WILL I BE COMPENSATED?**

In the event that you suffer an injury as a result of participating in this research, the Sport Injury Prevention Research Centre, the University of Calgary, Alberta Health Services or the Researchers will provide no compensation to you. You still have all your legal rights. Nothing said in this consent form alters your rights to seek damages.
SIGNATURES

Your signature on this form indicates that you have understood to your satisfaction the information regarding your participation in the research project and agree to participate as a participant. In no way does this waive your legal rights nor release the investigators, or involved institutions from their legal and professional responsibilities. You are free to withdraw from the study at any time without jeopardizing your health. If you have further questions concerning matters related to this research, please contact:

Gabriella Nasuti or Jamie Rishaug (research coordinators) or

Dr. Carolyn Emery (primary investigator)

If you have any questions concerning your rights as a possible participant in this research, please contact the Chair, Conjoint health Research Ethics Board, University of Calgary.

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<th>Participant Name</th>
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Par-Q forms are used to verify that participants may safely complete the physical activities contained in the study. The Par-Q consists of 7 yes/no questions. Participants are given a specific set of instruction:

**INSTRUCTIONS:**

This survey asks for your view about your knee. This information will help us keep track of how you feel about your knee and how well you are able to perform your usual activities. Answer every question by ticking the appropriate box, only one box for each question. If you are unsure about how to answer a question, please give the best answer you can.

1. Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?

2. Do you feel pain in your chest when you do physical activity?

3. In the past month, have you had chest pain when you were not doing physical activity?

4. Do you lose your balance because of dizziness or do you ever lose consciousness?

5. Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?

6. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?
7. Do you know of any other reason why you should not do physical activity?

Participants who answer yes to any of these questions are referred to a certified exercise physiologist to assess their potential risk. The physical portions of the testing may not proceed without the approval of a Canadian Society of Exercise Physiology CEP, or certified exercise physiologist. (http://www.csep.ca/en/membership/csep-cep)
III. APPENDIX III. ELISA TECHNIQUES

Jordan Laudon
Krawetz Laboratory [2015]

hCOMP ELISA Protocol (Wieslab)

Supplies:
hCOMP Quantitative ELISA Kit (Wieslab, REF COMP 200 RUO)
- Calibrators (10-80 ng/ml)
- Controls (1 & 2)
- Anti-COMP Serum
- Anti-COMP Conjugate
- PnPP Substrate
- Antigen-Coated well strips (12x8)
- Wash Buffer (30x)
- Dilution Buffer (1x)

Sterile multi-channel reagent trays
Round-bottom 96 well pre-incubation plates
Pipettes, all sizes
Pipette tips, all sizes
Multichannel pipette, 8 points (50-300 microliters)
Bulk 200 microliter pipette tips, no bevel
Kim Wipes
Shaker
Rocker
96-well Spectrophotometer
Ice & Black Foam Ice Container
Deionized Water

Part 1: Thawing, Dilutions & Incubations

1. Select which samples are to be tested. A single kit can sample up to 40 pairs in duplicate. If you have multiple samples from the same source, ensure that both samples are to be tested, in duplicate, on the same plate.
2. Fill ice bucket with ice, and place test samples immediately into ice from the -80 storage freezer. Wait until sample is at least half thawed before collecting any serum. (45-60 minutes after placement on ice).

3. Record the samples to be tested, and create a graphical representation of the 96-wells to be tested in your lab notebook. This representation should be identical in format to Table VII.1.

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<tr>
<td>C</td>
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<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Each box represents a 1x2 column for each sample test duplicate. C80-C10 represent calibration values. C1 & C2 are control samples (identical from each kit)

4. Label two new sets of 1.5mL tubes with the participant ID’s of each sample. One sample is for the dilutions to be assembled, the second set is for collection of 100 microliter samples for luminex testing.

5. Once samples are thawed, create a 1/50 dilution of serum sample and wash buffer.

6. Add 8 microliters of serum and 400 microliters of Dilution Buffer to one set of labeled tubes. Add the serum first, and ensure it is deposited in the very bottom of the tube. (If using synovial fluid, change to a 1/500 dilution program.)

7. Place diluted samples onto a rocker (minimum 5 minutes, no max) to ensure dilutions are properly mixed.

8. Collect 100 microliters of serum for each sample to be tested by luminex.

9. Now that both samples have been collected, return luminex aliquots to the -80 freezer, as well as the original test samples.
10. Place 75 microliters into each diluted sample into its corresponding duplicate locations on the pre-incubation plate.

11. Load 2 wells of a sterile triple-tray with the anti-COMP serum. Using the multi-channel pipette, add 75 microliters of anti-COMP serum to each well. Dispense the anti-COMP from the top of the well to prevent cross-contamination between wells.

12. Place the loaded pre-incubation plate on a shaker for 5 minutes, then place into a 4 degree refrigerator overnight. (Kit says 12-18 hours, up to 24 is acceptable)

**Day 2: Loading, Waiting, Washing, and Reading**

1. Remove the hCOMP kit from the fridge, allowing all reagents to return to room temperature.

2. Using the multi-channel pipette, place 100 microliters of Diluted Serum/anti-COMP from the pre-incubation plate into its corresponding location on the antigen-coated well strips. **Incubate 60 minutes.**

3. While the first incubation is running, prep a minimum of 3 boxes of disposable 200 microliter pipette tips (for wash steps). In addition, prepare 300 mL of wash buffer (10mL 30x wash, 290mL deionized water).

4. Once incubation is complete, wash each well 3 times with 200 microliters of wash buffer, using the multi-channel pipette.
   a. Each wash should aspirate the well fluid 5 times. After the first wash, remove all fluid from the well, and change pipette tips.
   b. Washes 2 and 3 can use the same pipette tips. Again, aspirating the fluid 5 times per wash. After the final wash, collect as much remaining fluid as possible from remaining wells.

5. After the wash, vigorously tap out the antigen-coated plate onto a kim wipe, to ensure all remaining fluids are excised.

6. Place 100 microliters of Conjugate (blue bottle) into each well. **Incubate 60 minutes.**

7. Wash 3 times with 200 microliters wash buffer, same as the previous wash.

8. Tap antigen-coated plate vigorously onto kim wipe.

9. Add 100 microliters of PnPP substrate to each well. **Incubate 50 +/- 10 minutes.**

10. While final incubation is occurring, prepare a 96-well spectrophotometer for use, measuring absorbance at 405nm.

11. Wipe down the bottom of the plate with a kim wipe, to ensure the clarity of the surface. Remove the top cover plate from the antigen-coated plate. Image the plate.

12. Export the resulting values to an excel file, and store the data. For redundancy, take a picture of the output to ensure the data is safely backed up.

13. Return all unused reagents to the hCOMP Kit, and store it in a 4 degree refrigerator.

14. Clean up work area, dispose of tips, wash glassware used in the protocol.
APPENDIX IV. KOOS QUESTIONNAIRE

The knee osteoarthritis outcome score questionnaire (KOOS) is a self-reported questionnaire regarding symptoms associated with knee dysfunction. Each question addresses a specific symptom, and allows the participant to record the frequency of occurrence for each symptom. Knee symptom frequency is recorded on an ordinal scale, as each question allows only a discrete set of responses. The exact questions and potential responses are shown below.

S1. Do you have swelling in your knee?
Never  Rarely  Sometimes  Often  Always

S2. Do you feel grinding, hear clicking or any other type of noise when your knee moves?
Never  Rarely  Sometimes  Often  Always

S3. Does your knee catch or hang up when moving?
Never  Rarely  Sometimes  Often  Always

S4. Can you straighten your knee fully?
Always  Often  Sometimes  Rarely  Never

S5. Can you bend your knee fully?
Always  Often  Sometimes  Rarely  Never
Results from the KOOS survey were recorded, and normalized such that an asymptomatic knee scored 100, with a score range of 0 to 100.
V. APPENDIX V. WESTERN BLOT TECHNIQUES

Western blotting is a procedure that allows the transfer and visualization of a protein of interest contained in a biological specimen. The protein of interest is spread across a gradient based on its molecular weight. The protein of interest, COMP, is tagged by a secondary antibody and fluoresced, allowing a visualization of intensity and distribution of the COMP content in each specimen by molecular weight.

Based on the early COMP outcomes from ELISA testing, participant samples were identified in the injured and uninjured cohorts expressing COMP levels at both extremes of the spectrum (high and low), as well as participants from both the injured and uninjured groups. Using this selection criterion, both extremes were represented, and qualitative assessments could be performed based on the blotting patterns produced in the experiment. Additionally, blotting allows qualitative assessments for differences between the COMP provided by participants, and to assess any differences between participants’ COMP and the human COMP provided as calibrators of the hCOMP ELISA kit (Wieslab) used in this study.

Western blots are produced through a series of several distinct steps. The first step is preparing and loading the acrylamide gel with serum samples and protein ladder. Acrylamide gels, when placed on an electrical gradient, allow proteins to move across the gel based on their molecular weight. The smallest proteins move the fastest, while larger proteins migrate more slowly. The protein ladder contains colored bands of proteins and specific molecular weights, producing a set of references to compare the protein of interest to.
After the proteins are embedded across the acrylamide gel, proteins are transferred off the gel onto nitrocellulose paper. The nitrocellulose paper is subjected to a series of blocking solutions to prepare the surface for binding, and incubated overnight in a COMP antibody that binds to the embedded COMP proteins. Finally, the nitrocellulose paper is incubated in a secondary antibody which binds to the COMP-antibody conjugate. This complex is known to fluoresce at the 680nm wavelength, where the final images of the blots are produced.

Invariably, Western Blotting procedures must be tailored to the specific protein of interest. Therefore, the lab protocol employed has been reproduced, with specific notes developed during testing. The protocol is written to produce two sister gels, meaning two unique gels produced during the same protocol.

Reagents & Equipment:
- 10x Transfer Buffer (288g glycine, 60.4g tri-HCl, 1.8L deionized H₂O)
- 1x Transfer Buffer (100 mL 10x Transfer buffer, 100mL methanol, 800mL deionized H₂O)
- 1x TGS Running Buffer (6.04g tris-HCl, 28.8g glycine, 2g SDS, 2L deionized H₂O)
- 1x PBS (Phosphate Buffered Solution)
- Tween-20
- Protein Ladder (Bio-rad Kaleidoscope, Cat #161-0375)
- COMP antibody (from Wieslab hCOMP ELISA, used in COMP evaluations)
- COMP Antiserum (Biorad, rabbit 680 Cat #170-6515)
- Acrylamide gels (Bio-rad, 10% acrylamide, 15 lanes)
- Bromophenol Blue
- Cell lysis buffer (Bio-rad, 100mL distilled H₂O, .5M Tris-HCl, .5M glycerol, 10% (w/v) SDS, 10% (w/v) 2 b-mercaptoethanol, .05% (w/v) bromo blue)
- Filter Paper
- Nitrocellulose Paper
- Transfer Buffer
- Powdered Milk
- Serum Samples
- Bio-rad Protein ii XI Cell (Western Blotting Chamber)

Steps:
Preparing and Loading the Gel

1. Before we can begin loading the gels, samples must be prepared. There are 28 lanes available for testing. Select 14 samples to test.
2. Thaw samples from -80 C to 0 C, by placing them in a container of crushed ice. Thaw will take around 40 minutes. Samples should be at least half melted before collecting sample.
3. Collect 10 microliters of serum from each sample. (Collect in 1.5ml tubes)
4. Add 90 microliters of “Cell lysis buffer w/bromophenol blue) ***Get ratio of lysis buffer and bromo blue from Roman***
5. Turn on heat rack, high setting.
6. Sonicate each sample to ensure proper mixing. Centrifuge ~10 seconds to collect sample in tube bottom.
7. Boil samples in heat rack (use thermometer) at 95 C for 5 minutes. Leave at room temperature for 5 minutes. (Use boiling caps if 1.5mL tubes are pop tops)
8. Turn off heat rack.
9. Assemble 2x acrylamide gels in electrophoresis rack. Make sure to remove green strip from bottom prior to running the gels. Failure to do so will ruin the gels.
10. Fill outer rack area to the “2 gel” line with 1x TGS. Fill the inner area of the rack to the tip of the gel combs in 1x TGS.
11. Record order of samples to be inserted in gel. Make sure it is identical for both gels.
12. Begin loading samples. Load 5 microliters Kaleidoscope Protein Ladder into the leftmost lane. Load 13 microliters to each subsequent well.
13. Ensure gels are loaded Black-Black and Red-Red.
14. Plug in electrophoresis machine, 40 amps and 100v. Ensure bubbles are floating from bottom of gels, and that amps stay in the 30-40 range. Amps <5 indicates a problem.
15. Allow current to run until bromophenol blue has reached the white-green divide at bottom of gel, then discontinue the current.

Transfer Gel to Nitrocellulose Paper

1. Set up transfer sandwich. Order:
   Black Plastic Backing
   Black Mesh Sponge
   Filter Paper
   Acrylamide Gel (from previous steps)
   Nitrocellulose paper (target of transfer steps)
   Filter Paper
   Black Mesh Sponge
   White Plastic Casing
   Rabbit secondary antibody680
   10x TGS
   1x PBS
Note: When Setting up transfer sandwich, use tweezers for all aspects! Touching nitro paper, even with gloves, will damage the test quality significantly. Filter paper and nitro paper must be cut to size, aim for an area slightly smaller than the gel itself.

2. Insert transfer sandwich into empty electrophoresis container. Insert an ice pack into the open space. Failure to add ice pack will result in catastrophic meltdown.
3. Fill to the “blotting” line with transfer buffer, which should not be confused with running buffer.
4. Fit black-black and red-red, connect the power.
5. Run for exactly 1 hour at 100V, 100 amps. No more, no less.
6. The nitro paper now has our proteins! Do not remove from sandwich until blocking container and blocking solution is prepared. [pipette boxes work well for this]

**Blocking & Applying Antibodies**

1. Before adding any antibodies, block paper for 60 minutes in 1x PBS w/3% milk. Incubate on a shaker box at room temperature. (15mL per paper). [1xPBS w/3% milk created w/ 50mL 1x PBS + 1.5g powdered milk)
2. Create antibody/block mix. Our first attempt included high (15mL block + 500 microliter anti-COMP serum, 15mL block + 250 microliter anti-COMP serum [from Wieslab hCOMP kit]
3. Pour out blocks, and add antibody/block mix. Shake 30-45 minutes at room temp.
4. Place papers, still in the block, into the 4 C fridge overnight/weekend/til tomorrow.

**Day Two: Blocking, Applying Conjugate Antibody, and Reading the Nitro Paper**

1. Remove blocking boxes from 4 C fridge, and place on shaker ~40 minutes, until returned to room temp.
2. Drain off blocking solution, and wash 3x for 5 minutes using PBST (50mL of PBS w/250 microliters Tween-20). Use ~12 microliters/wash/paper. Keep boxes on shaker.
3. Block for 1 hour in tween blocking solution (3% milk PBST). Keep boxes on shaker.
4. Mix 20 mL of 3% milk PBST with 20 microliters of Anti-rabbit secondary antibody 680. Drain PBST, and add blocking/secondary mix. (10ml per paper). Incubate 1 hour on shaker.
5. Wash 3 times in 1x PBST (no milk), 5 minutes each. Keep boxes on shaker.
6. Gel is now ready for reading on scanner. Absorbance should be set to the fluorescence of the secondary antibody (680nm).

The protocol is now complete, and the image can be captured at 680nm. Experiences in the lab suggest that in the event of a blurry or poorly contrasting image, additional washes of the
nitrocellulose surface in PBST may improve the contrast within the image. However, too much additional washing may purge the fluorescing complex from the paper entirely, so caution is advised at this point in the procedure.
VI. APPENDIX VI. INTERPRETING ELISA RESULTS

Requirements: Excel, Statistical Software (STATA Preferred)

Interpreting the results of an hCOMP ELISA Kit (Wieslab) requires a number of steps. A regression curve must be calculated based on the calibrator samples, and confirmed by assessing the outputs on kit samples provided in each experiment. Duplicate points must be verified according to our \textit{a priori} cutoff of variation. Finally, the curve may be applied, and the initial serum dilutions accounted for. In the end, each data point will have a mean COMP value, and an associated coefficient of variation (CV). Each step will be explained and demonstrated.

Finding the Data

The data from each experiment should be available in a 12x8 excel file, as shown.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
<th>L</th>
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<td>1.487</td>
<td>1.061</td>
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<td>1.509</td>
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<td>1.006</td>
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</table>

Columns A & B contain the calibrator and control sample values.

A1-2 = 80ng/ml
A3-4 = 60ng/ml
A5-6 = 40ng/ml
A7-8 = 30ng/ml
B1-2 = 20ng/ml
B3-4 = 10ng/ml
Using these values, a simple excel sheet can prepare the data for regression analysis. In 3 columns, place the concentration (80, 60, 40, etc) next to the absorbance from each well. Calculate the absorbance squared in an adjacent column, as shown.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
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<tbody>
<tr>
<td>1</td>
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<td>Abs</td>
</tr>
<tr>
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<td>80</td>
<td>0.341</td>
</tr>
<tr>
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<td>40</td>
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<td>30</td>
<td>0.989</td>
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<td>30</td>
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<td>20</td>
<td>1.444</td>
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<tr>
<td>12</td>
<td>10</td>
<td>1.59</td>
</tr>
<tr>
<td>13</td>
<td>10</td>
<td>1.616</td>
</tr>
</tbody>
</table>

Save this excel sheet, and import it into STATA. (Any statistical software package will do).

**Creating The Regression Curve**

If using STATA, ensure that when imported, the top column is tagged as variable names, or the software will not cooperate. Create a linear regression using the variables created in the table. “Conc”, or concentration, is the dependent variable. “Abs” and “AbsSquare” are the independent variables. While the manipulation is done via “linear regression”, the fact that one term is a squared version of the other means this is in fact a second order regression. Investigation into the curve between absorption and concentration will support the fact that the relationship is not strictly linear, and is better served by a second order regression.

Record the output from STATA, and create a formula for the regression in \( y=ax^2+bx+c \) format.
y = 43.56x^2 - 132.64x + 114.87; y = Concentration, x = absorbance

Now that the regression curve is created, we can determine the true concentrations of COMP in each sample. However, we must ensure that each duplicate test has a Coefficient of Variation (CV) that does not exceed 10%, our a priori cutoff point.

\[
SD = \sqrt{\frac{\sum (x_1 - x_2)^2}{2n}}
\]
\[
Mean = \frac{\sum (x_1 + x_2)}{2n}
\]
\[
CV(\%) = 100 \times \frac{SD}{Mean}
\]

Using these formulas\(^1\), a Coefficient of Variation can be derived for the duplicate test values associated with each sample. See the attached excel spreadsheet “COMPCVCalculations” for examples of necessary coding to produce these values.

***Note*** Upon completion, these same formulas can come up with aggregate statistics for the COMP ELISA’s overall performance.
Duplicate values whose CV exceeds 10% must be flagged and retested in duplicate. (More on this later)

**Calculating the True Values**

Now that we have our regression curve, and have tested for unreliable duplicate samples, we can calculate the actual concentrations of COMP in the tested serum. In excel, plug each individual absorbance reading into the regression formula. Most values should fall between 10 and 80 nanograms. Now we must undo the effects of our 1:50 dilution from the beginning of the hCOMP protocol. To do this, we must multiply each outcome by a factor of 51. Values typically will fall between 600 and 4000 ng/mL at this time.

For each duplicate sample, combine the final output of both wells to create an average score for each. Associate each averaged value with its original participant and record.

**Handling High CV Results**

On occasion (~10% of results) duplicate results will have absorbance with a Coefficient of Variation beyond 10%. In these cases, the results are still calculated, but an additional duplicate test of the sample must be performed. Assuming the new results have an acceptable CV, compare the new values with those produced by the original test. If a clear outlier is identified from the initial test, drop the outlier, and instead take the average of the three remaining outcomes.

**Comparisons to Other hCOMP Reported Values**
A variety of academic papers have reported baseline serum COMP concentrations, and statistical 
descriptions of variation, such as the Coefficient of Variation. A variety of sources$^{2,3,4,5}$ have 
found their composite CV to be at or under 5% across all samples. Under the statistical methods 
put forth here, the CV of the data produced is comparable to that of other studies published with 
the same hCOMP ELISA kit.

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