Anti-Gravity Exercise, Creatine Supplementation, and Serum S100A8/A9 in Knee Osteoarthritis

BY

PENGYU ZHANG

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Department of Human Anatomy and Cell Science

Rady Faculty of Health Sciences

University of Manitoba

Winnipeg, Canada

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TABLE OF ABBREVIATIONS

ANOVA	Analysis of variance
ADL	Activities of Daily Living
BMI	Body Mass Index
COMP	Cartilage Oligomeric Matrix Protein
ELISA	Enzyme-Linked Immuno-Sorbent Assay
FWB	Full Weight Bearing
HR	Heart Rate
IL	Interleukin
K&L	Kellgren & Lawrence grading scale
KOOS	Knee Injury and Osteoarthritis Outcome Score
LBPP	Lower Body Positive Pressure
NSAID	Non-Steroidal Anti-Inflammatory Drug
OA	Osteoarthritis
OR	Odds Ratio
QOL	Quality of Life
Sport/Rec	Sports and Recreational Activities
TNF	Tumor Necrosis Factor
TKA	Total Knee Arthroplasty
VAS	Visual Analog Scale
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index

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ABSTRACT

Knee osteoarthritis (OA) is a degenerative joint disease defined by the breakdown of cartilage with pain, stiffness, and swelling. An inflammatory process could be involved during the progression, and S100A8/A9 is considered a hallmark cytokine. With Lower Body Positive Pressure (LBPP) anti-gravity treadmill exercise, knee OA patients can walk with normal gait. Creatine monohydrate supplementation can decrease inflammation and improve the muscle strength. The central hypotheses stated was: inflammation associated with knee OA is related to serum S100A8/A9 level. The aims of this study focused on the effect of interventions and the level of serum S100A8/A9.

Twenty-six (26) overweight participants with a mean age of 57 years were randomized into LBPP exercise, creatine supplementation, and control groups. KOOS scores, BMI, ROM, pain VAS score, and serum biomarkers were assessed. Statistical analysis indicated LBPP and Creatine could help to improve the function and disability. However the biomarkers were in normal range and not correlated to interventions.

1. INTRODUCTION

Osteoarthritis (OA), also known as degenerative arthritis or degenerative joint disease, is a prevalent joint disorder that is accompanied by a chronic inflammatory process (Frye, Shmalberg, & Wakshlag, 2016). It is referred to as a structural abnormality associated with the degradation of joints, especially the articular cartilage. Although OA can occur in any joint, it most commonly affects weight bearing joints such as spine, hip, and the knee. Its symptoms include progressive pain, stiffness, and joint effusion, which result in ever increasing disability ("The Basics of Osteoarthritis," n.d.).

In knee OA, all three compartments of the knee joint (medial, lateral and patellofemoral compartments) can be affected (Bartz & Laudicina, 2005; Englund, Guermazi, & Lohmander, 2009; Huber, Trattnig, & Lintner, 2000). This disease normally progresses slowly over the years, reducing the quality of life of millions of people in North America ("Data: Self-reported prevalence and number with arthritis in Canada, by province and territories, 2005," n.d.; O'Donnell et al., 2015). The pathophysiological mechanisms underlying this disease still remain unclear.

Inflammation plays a role in knee OA (Frye et al., 2016). The general description of inflammation is stated as a localized physical condition that occurs as a result of injury or infection. Its symptoms include redness, swelling, heat, pain, and dysfunctions. Swelling and pain are the two major symptoms in knee OA (Nuki, 1999). Beyond this, the process of knee OA is influenced by chronic inflammation. Cytokines associated with inflammation may affect the normal biological function of articular cartilage (Robinson et al., 2016).

Currently, clinical OA diagnosis and evaluation are made by radiographic examination and questionnaire. Kellgren and Lawrence Classification(K&L) via X-ray is the most common method of examination used to rate the extent or degree of knee joint degeneration (Schiphof, Boers, & Bierma-Zeinstra, 2008); KOOS (Knee injury and Osteoarthritis Outcome Score) is a subjective and individual specific questionnaire used to analyze a patients' rating of knee function and pain to reflect their daily function and disability, and has been used in screening and following-up (N. J. Collins et al., 2016).

S100A8/A9 is a biomarker that has been used for quantifying inflammation associated with inflammatory conditions in chronic bowel disease (CBD), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) (Clohessy & Golden, 1995; Gross, Sin, Barraclough, & Rudland, 2014). Serum S100A8/A9 is considered as a biomarker to quantify inflammation in mild to moderate knee OA, and may provide information on the inflammatory status of the knee joint (P. L. E. M. van Lent et al., 2012).

LBPP (Lower Body Positive Pressure) exercise and Creatine supplementation are the two interventions in this study. LBPP exercise facilitates anti-gravity training and rehabilitation and was first developed by NASA (Knapp & Evans, 1996). Moderate LBPP exercise for knee OA patients has been proven to significantly improve the strength, function and disability (Peeler, Christian, Cooper, Leiter, & MacDonald, 2015; Takacs, Anderson, Leiter, MacDonald, & Peeler, 2013). Creatine supplementation as a non-essential nutrient has the ability to decrease the level of inflammatory biomarkers and enhance muscle strength (Kitzenberg, Colgan, & Glover, 2016). Its safety has been confirmed in general population (Bizzarini & De Angelis, 2004).

In summary, our working theory was based on the mechanism and evaluation of knee OA. We assumed that knee OA is developed by inflammation and mechanical stress, which could be improved by Creatine supplementation and LBPP exercise. In the present investigation, the biomarker S100A8/A9 was used as an objective method to quantify inflammation associated with knee OA disease progression, and its results were compared with subjective evaluation of knee OA patients via the KOOS questionnaire.

The hypothesis for this study was: inflammation associated with knee OA is related to serum S100A8/A9 level. The primary aims of this study include: 1. To quantify the level of serum S100A8/A9 concentration in knee OA patients, 2. To determine whether serum S100A8/A9 levels can be influenced by the LBPP exercise or Creatine supplementation, and 3. To determine whether serum S100A8/A9 level is related to knee OA severity (K&L) or the symptoms (KOOS) in knee OA patients.

2. REVIEW OF THE LITERATURE

2.1 Anatomical Structure of Knee Joint

2.1.1 Introduction

The knee joint is one of the most complicated joints of the human body. The stability of the knee depends on ligamentous restraint, thigh muscle strength, and congruent articulation between the femoral condyles and the tibial plateau. According to a description by Vingard (Vingård, 1996), the mechanical function of the knee is supported by three main groups of ligaments: 1. lateral collateral ligament, 2. medial collateral ligament, and 3. anterior and posterior cruciate ligaments. The movement of the knee is powered by thigh muscles that cross the knee joint, including the patellar tendon on the anterior aspect of knee. The range of knee movement is restricted by articular cartilage. The articular cavity is surrounded by a synovial capsule, which is filled with synovial fluid secreted by peripheral soft tissue for lubrication. Each of these anatomical structures has their own unique metabolism, but they work together in unison to ensure effective function of the knee joint. As such, any mechanical or physical change to joint structures can disturb joint homeostasis, and result in functional disorder.

	Function	Role in knee OA
Muscle	Power movements	Weakness found in patients
Cartilage	Smooth surface of joint	lesion is the pathological progression
Synovium	Nutrition, lubrication (fluid)	Swelling relating to inflammation
Ligament	Restrict range of movement	Injury induces knee OA
Meniscus	Load transmission, shock absorption	Degeneration and injury induces knee OA

2.1.2 Muscles & Movements

The general function of muscles is to power movements, and act as an antagonist to limit excessive or abnormal movements (A. V. Hill, 1960). At the knee joint, the movements include flexion/extension, and medial/lateral rotation. The Quadriceps femoris muscle group is located in the front of thigh and inserts to the tibial tuberosity, and powers knee extension. Its main antagonist muscles which flex the knee are the hamstrings located in the posterior thigh, and the gastrocnemius muscle in the lower leg. The popliteus muscle rotates the tibia on the femur to unlock the knee during flexion ("Gray, Henry. 1918. Anatomy of the Human Body," n.d.). In a knee OA population, quadriceps weakness is one of the most common findings, being 20 – 40% lower than healthy population (Bennell, Hunt, Wrigley, Lim, & Hinman, 2008). The compensation of the weakness of muscles may also lead to more stress on the meniscus. It has been demonstrated that injury of meniscus is one of the causes of knee OA (Englund, 2009; Englund et al., 2009).

Knee movement is also limited in knee OA patients. Generally, the normal range of lower limbs' joints is described as: Hip Flexion 90–135°, Extension 10-30°; Knee Flexion 130–140°, Extension 5–10°; Ankle Plantar flexion 40–50°, Dorsi flexion 15–20° (Medicine, 2013). The range of motion (ROM) may be significantly reduced in knee OA patients (Field, 2016). However, the limited ROM does not correspond to muscle weakness (Bennell et al., 2008). The reason for the reduction in ROM still remains unknown.

2.1.3 Cartilage

Cartilage is central to the pathological observations in knee OA (Brandt, Dieppe, & Radin, 2009; Huber et al., 2000). Different from bone tissue, cartilage is described as a relative elastic tissue composed of chondrocytes and collagenous extracellular matrix. The chondrocytes are the factory of the extracellular matrix. However, any lesion on the cartilage is never perfectly repaired: the hyaline cartilage will be replaced by fibrocartilage scar tissue, which is made of a rougher surface. Furthermore, no blood vessel or nerves can penetrate into the cartilage, and this increases the difficulty of cartilage repairing. The nutrition differs between the synovial fluid and subchondral areas, and is influenced by movement of joint (Brandt et al., 2009; Huber et al., 2000).

2.1.4 Synovial Capsule

A synovial capsule covers the knee and secretes synovial fluid for the nutrition and lubrication of the joint. Generally, the outer fibrous membrane is composed of dense connective tissue and holds the shape of synovial capsule. The inner synovial membrane produces the fluid, and has a stronger correlation with inflammation (Mahler et al., 2015). The type-A cells, differentiated from macrophages, can remove debris floating inside the synovial fluid to keep an appropriate nature for joint movement. Type-B cells are fibroblast capable of secreting hyaluronan and other components of the synovial fluid. Under inflammatory conditions, the balance between these two cell types is disrupted. Macrophage recruitment can lead to a thickened synovial membrane, while type-B cells are less effective at producing hyaluronan for lubrication, and can even digest the cartilage extracellular matrix with enzymes (Brandt et al., 2009; Man & Mologhianu, 2014; X. Wang, Hunter, Jin, & Ding, n.d.).

2.1.5 Ligaments & Meniscus

The ligaments and menisci are also important structures in the knee joint, but less likely to be involved in OA. There are two main groups of ligaments (collaterals and cruciates) that passively limit the range of motion at the knee joint (Bartz & Laudicina, 2005). The property of ligaments is different from muscle tissues, which are much more elastic and voluntary. Their main function is to restrict rotation and anterior-posterior movement. The tear of medial collateral ligament is commonly associated with injuries of medial meniscus.

Menisci are C-shaped, fibro-cartilaginous structures between the femoral condyles and tibial plateau inside the synovial capsule. Its function includes load transmission, shock absorption, and promoting the flow of synovial fluid. They covers approximate 70% of the area of the tibial plateau articular surface (Raj & Bubnis, 2017), and up to 70% load to tibial plateau in extension and 90% of load during knee flexion is transmitted through meniscus. Injury of meniscus results in difficulty distributing mechanical stress (Greis, Bardana, Holmstrom, & Burks, 2002). The mechanical properties of the meniscus can change with aging. Not only will collagens degrade overtime, the function of cells will also promote the process of dysfunction (Tsujii, Nakamura, & Horibe, 2017).

2.2 Knee Osteoarthritis

2.2.1 Pathogenesis & Risk Factors

Knee OA is classified as either primary or secondary based on any known risk factor in the medical history. Generally, if there is no obvious related medical concerns, knee OA will be diagnosed as primary (Nuki, 1999; Ewa M. Roos & Lohmander, 2003). The genetic, congenital, or developmental reasons are considered, although these factors are unlikely to be the main cause of knee OA (Y. Zhang & Jordan, 2008). There are various risk factors, including congenital joint disorders, diabetes, inflammatory diseases, injuries, and obesity that may lead to the development of secondary OA (Nuki, 1999). The primary and secondary classifications attempt to guide the treatments for knee OA based on the pathogenesis.

	Risk factors
Primary knee OA	Genetic, Congenital, unknown
Secondary knee OA	Joint disorder, Diabetes, Inflammatory disease, Injury, Obesity

The American College of Rheumatology ("Diseases and Conditions Osteoarthritis," n.d.) states that both biochemical changes and mechanical stress can play a role during the progression of knee OA. With the increased understanding of the pathophysiological process of knee OA, the discrimination between primary and secondary knee OA has been blurred (Arden & Nevitt, 2006), and this classification becomes less meaningful both in clinic and research.

2.2.2 Symptoms

Knee OA is classified as either symptomatic or asymptomatic in clinical settings. The main symptomatic complaint of most knee OA patients is pain in the knee joint

which motivates them to seek medical attention for diagnostic purposes (Hunter & Lo, 2008; Y. Zhang & Jordan, 2008). However, there are also a group of patients without symptoms until the terminal stage requiring knee replacement. No previous studies have revealed the reason why patients may not experience symptoms, and no pathological differences were reported between symptomatic or asymptomatic knee OA.

Pain is always the first symptom noticed in most knee OA cases. Although pain is one of the symptoms of inflammation, it is still unclear how pain in knee OA happens and how severe it is (C. L. Hill et al., 2001; Catherine L. Hill et al., 2007). Most clinicians believe that the pain comes from the peripheral tissues, which is induced by swelling or stimulated by chemical substances. However, in late stage OA patients whose knee cartilage has almost disappeared completely, some of them felt no pain at all, whereas others suffer more severe pain and lost the ability to walk.

2.2.3 Signs and Physical Function

The signs of knee OA include osteophytes, narrowed joint space, and necrosis at the subchondral bone (Kellgren & Lawrence, 1957; Y. Zhang & Jordan, 2008). According to the unknown etiology of knee OA, there is still no robust explanation regarding formation of osteophytes and subsequent changes. Osteophytes, the most representative characteristic of mild to moderate knee OA, are formed due to bone remodeling, which is directed by osteoblasts and osteoclasts. It is not known whether the formation of osteophytes is due to a harmful inflammatory reaction, or a protective response to excessive load at the knee joint (Barsa, Novák, Souček, Maršík, & Suchomel, 2011; Waldstein, Kasparek, Faschingbauer, Windhager, & Boettner, 2017, p.). Even

though it is not known why osteophytes form, they are still used as evidence of knee OA on X-ray examination. Besides considering symptomology, clinicians simultaneously use these signs to grade the severity of OA, and provide further treatment.

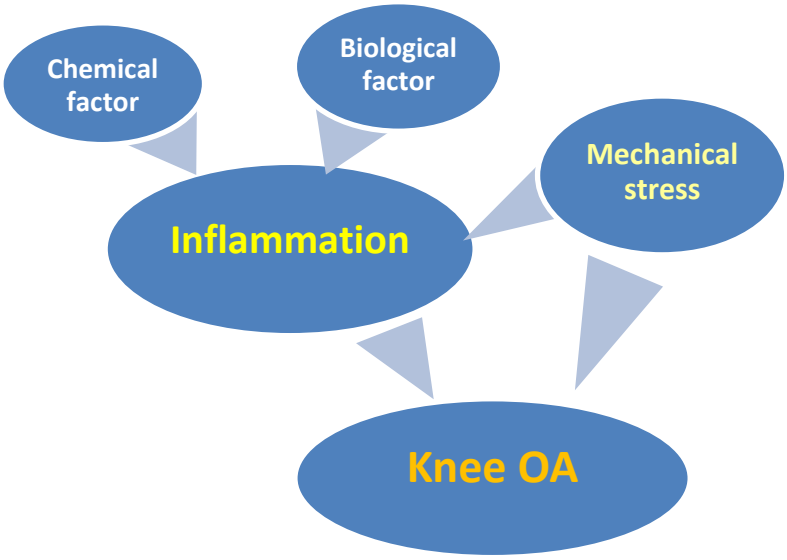
Physical function, such as the range of motion about the joint and muscle strength, are also parameters to evaluate the status of patients (Jinks, Jordan, & Croft, 2007). Generally, weakness of quadriceps has been confirmed in knee OA patients. Muscle mass and muscle strength both deteriorate in the population. Recent studies also suggest that dysfunction of the gluteal medius is also a possible trigger of knee OA: this muscle may help maintain stability at the hip joint, thus avoiding unnecessary compensatory movements at knee joint (Steinberg, Dar, Dunlop, & Gaida, 2017). Overall, previous studies have illustrated that the physical function is dramatically reduced in a knee OA population, and it can be improved with proper treatment.

2.2.4 Inflammation

Inflammation is typically described as a part of a complex biological response to a harmful stimulus by the tissues. Chemical, mechanical, and biological factors are all capable of inducing inflammation. Inflammation can be classified as acute or chronic. The symptoms of acute inflammation include pain, heat, redness, swelling, and dysfunction, while chronic inflammation is more moderate and it involves subtle but permanent changes within the body (Ferraccioli et al., 2010; Stríz & Trebichavský, 2004). Inflammation is considered a protective process which is mediated by molecules of the innate immune system (Goyette & Geczy, 2011). The molecules that direct the innate immune inflammatory reaction can generally be divided into pro-inflammatory cytokines

or anti-inflammatory cytokines. Each of these molecules have multiple functions and are at work in different cell types under various settings during the process of inflammation (Dmitrieva, Shilovskiy, Khaitov, & Grivennikov, 2016; Garcia-Arias et al., 2013; Greene & Loeser, 2015; Ometto et al., 2017).

Inflammation is a key component during the development of knee OA. Although inflammation may lead to pain, swelling, synovial membrane thickening, and other symptoms, it is a double-edged sword in our body: it is a generally protective process in most situations, but also a pathological process if it gets out of control.



2.3 Cytokines & Biomarkers

2.3.1 Tissues / Cells inside knee joint

2.3.1.1 Chondrocytes

The two major components in the progression of knee OA are chondrocyte and cartilage. Chondrocytes are the only cell type in the cartilage, and thus the only source of

cartilage matrix production (Huber et al., 2000). Originally, chondrocytes are differentiated from mesenchymal stem cells (MSC), which are also the precursors of osteoblasts. Chondrocytes secrete extracellular matrix (ECM) to compose the hyaline articular cartilage. ECM is quite heterogeneous, and contains proteoglycans, glycosaminoglycan, and fibers. The components and chondrocytes are not evenly distributed across the different layers of cartilage (Huber et al., 2000; P. L. van Lent et al., 2008). One hypothesis about the pathogenesis of knee OA states that the chondrocytes apoptosis or autophagy is the starting point of cartilage damage; the death of chondrocytes can be triggered by inflammatory cytokines, and a vicious circle could be started. However, in knee OA observed *in vivo*, the inflammatory markers and damaged chondrocytes are present simultaneously, which fails to demonstrate the causal ("chicken and egg") relationship between cell death and inflammation.

To our knowledge, there is no previous study published on the concentration of S100A8/A9 in chondrocytes. As a result, we cannot conclude how much S100A8/A9 in the serum or synovial fluid came from chondrocytes. The source of S100A8/A9 would be important to deduce its possible function from a clinical viewpoint. Future research should be directed at evaluating how much S100A8/A9 is generated by chondrocytes, as this may help researchers to better understand its role in inflammation associated with knee OA.

2.3.1.2 Macrophages

Macrophages, which are a subtype of neutrophils, play a key role during the progression of knee OA. There are a group of tissue macrophages on the synovial

membrane known as Type-A cells. When inflammation happens, more macrophages will migrate into the synovial membrane (Catalán et al., 2011). The main functions of macrophage are anti-inflammatory, which help to clear up cell debris at the inflammation site, and surrounding the inflammatory site. A thickened synovial capsule secondary to the infiltration of macrophages can be observed in knee OA. The other aspect of macrophages is the accumulation of cytokines. Both pro-inflammatory and anti-inflammatory cytokines can be secreted by macrophages (Minciullo et al., 2016). Several representative cytokines will be discussed below.

2.3.2 Biomarkers from Tissues / Cells (Biochemistry)

2.3.2.1 CRP

C-reactive protein (CRP), discovered over 80 years ago, is one of the most representative inflammatory biomarkers. It is a pentameric protein which is widely used as a biomarker for inflammation (Thompson, Pepys, & Wood, 1999). It is predominantly produced by hepatocytes upon stimulation of interleukin-6 secreted from T cells and macrophages. After binding to its receptor, CD32/CD64, CRP activates the NF-κB signaling pathway and induces inflammation. Many studies have reported that besides inflammation, high CRP levels also correlate with risks of epithelial cancers (such as lung, liver, and breast cancers) and aging-related diseases (kidney diseases, diabetes mellitus, and cardiovascular diseases) (Lu, Ouyang, & Huang, 2006). In clinical practices, CRP level has been widely used as an indicator for pulmonary embolism (Ateş et al., 2016) and the risk for developing cardiovascular diseases (Danesh et al., 2004).

CRP is one of the biomarkers used to determine the systemic inflammation level. Acute inflammation can elevate the CRP concentration to 1 - 1000 mg/L (Wu, Potempa, El Kebir, & Filep, 2015). Similarly, interleukins (ILs) and tumor necrosis factors (TNFs) are also related to pro- or anti-inflammatory functions (Minciullo et al., 2016) and shows correlation to the level of inflammation.

2.3.2.2 Tumor Necrosis Factor

Tumor necrosis factor (TNF) superfamily consists of 19 cytokines that bind their receptors and induce cell apoptosis (Bazan, 1993). TNF-α is the most studied member of this superfamily, and is mainly produced by monocytes and macrophages. It plays an important role in immune processes as well as in cell programmed death (apoptosis and autophagy). Pasparakis et al. (1996) reported that TNF-α is necessary for the function of lymphoid organs in defeating pathogens (Pasparakis, Alexopoulou, Douni, & Kollias, 1996; Pasparakis et al., 1996).

2.3.2.3 Interleukin

Interleukins (ILs) are a supergroup of cytokines first observed in the secretions of leukocytes. Most of the interleukins are synthesized by helper CD4 T lymphocytes, while some others are synthesized by macrophages, and monocytes. The IL-1 family consists of a group of cytokines, which have been reported to be important in the training of innate immunity, and thus the initial host defense to pathogens and toxic substances (Moorlag, Röring, Joosten, & Netea, 2018). There are 11 reported members of IL-1 family, which

include 7 pro-inflammatory agonists and 4 anti-inflammatory antagonists (Palomo, Dietrich, Martin, Palmer, & Gabay, 2015). The levels of IL-1 cytokines are relatively low in host circulation; however, upon infection, they induce robust inflammatory responses. As a member of the IL-1 family, IL-1β is an important mediator in inflammatory responses. The proproteins of IL-1β are secreted by macrophages, and then they are processed and activated by caspase 1. IL-6 is another family commonly considered with inflammation. It is a glycoprotein which induces the maturation of immunoglobulin-producing cells (Hirano et al., 1986). IL-6 is secreted by T cells, B cells, and macrophages. The production of IL-6 is activated upon antigen stimulation (including viruses, bacteria, and chemokines).

2.3.2.4 Cartilage Oligomeric Matrix Protein

Cartilage Oligomeric Matrix Protein (COMP) is a chondrocyte extracellular matrix protein mainly existing in cartilage, and also is found in other musculoskeletal tissues. It is composed of five glycoprotein subunits (Oldberg, Antonsson, Lindblom, & Heinegård, 1992). The molecular weight of COMP is 82, 860 Da, which is much larger than S100A8/A9 (23,945 Da). In OA , COMP can be detected in serum, urine, and synovial fluid, and the concentration of COMP parallels the progression of early stage knee OA (Sharif, Kirwan, Elson, Granell, & Clarke, 2004). In both OA and RA, the concentrations of COMP are very similar and do not correlate to the level of inflammation (Wisłowska & Jabłońska, 2005). Longitudinal studies in an elderly population revealed an increase in the proportion of COMP correlated with increased risk

of hip OA (Chaganti et al., 2008). The biological functions of COMP may relate to skeletal development and the down-regulation of apoptosis (Barnes & Janecke, 2017), but its mechanism in these cellular processes is still unknown.

Unlike the systemic inflammatory cytokines listed above, COMP is one of the specific markers to evaluate the mechanical stress pressure on joint cartilage. A positive relationships between mechanical stress and osteoarthritis has been illustrated in several previous studies (Greene & Loeser, 2015). The concentration of serum COMP is influenced by multiple factors, including age, gender, and ethnicity. In healthy African Americans, COMP concentration is elevated with aging; in healthy Caucasians, the concentration in 55 - 64 year old group is the lowest compared to those 45-54 and older than 64. Otherwise, the serum COMP concentration is increased with the severity of K&L grades (Jordan et al., 2003). Males always present a higher serum COMP concentration than female within the same age group (Jordan et al., 2003; Perruccio et al., 2017). However, a more recent study in 2015 suggested that the serum COMP level was independent of age and BMI (Kluzek et al., 2015). There is an interesting finding published in 2016 that stated: the serum COMP concentration was increased after 30 minutes of running; while synovial COMP concentration was decreased (Hyldahl et al., 2016). The mechanism remains unknown: the synovial COMP could be pumped out from joint cavity to blood, or the increased serum COMP could come from muscle.

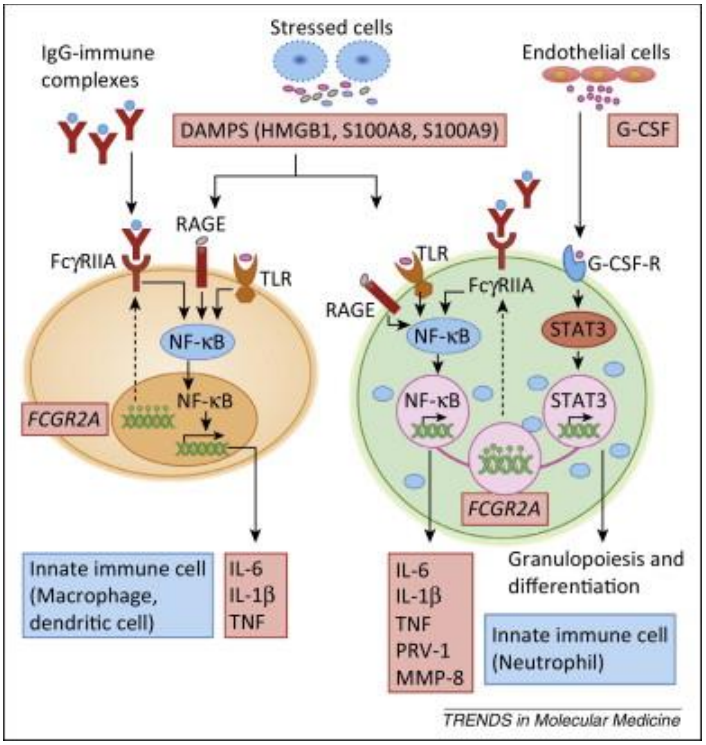
2.3.2.5 S100A8/A9

S100A8/A9 is a protein polymer, that is known alternatively as Calprotectin, MRP8/14 or Leuko-protein L1 (Berntzen, Olmez, Fagerhol, & Munthe, 1991; Emrani et

al., 2008). It is part of a large group of immune markers called Damage Associate Molecular Patterns (DAMPs) (Foell, Wittkowski, Vogl, & Roth, 2007). S100A8/A9 is composed of two subunits: S100A8 and S100A9, and together they form two helix-loop-helix structure to catch Calcium or Zinc (Perera, McNeil, & Geczy, 2010). The pro-inflammatory property is expressed only when the two subunits combine together. It is said that S100A8 plays the main role in producing inflammation, and S100A9 assists S100A8 to keep its stability and competent. Calcium or Zinc binding to the protein complex changes its configuration to recognize target cells and mediate functions: smooth muscle contraction, cell cycle, apoptosis, autophagy, and inflammation (Goyette & Geczy, 2011). The pathways that S100A8/A9 participate in include (HMGB1) - RAGE – TLR-4, Bcl-2, NF-κB – TNF-α, Fcγ family, among others (Foell, Wittkowski, & Roth, 2007).

Due to its chemical structure, S100A8/A9 was initially identified as an anti-microbial protein secreted by leukocytes, and given the name Leuko-protein L1 (Berntzen et al., 1991, p. 1). Later, its complex was found to contain two protein subunits, called myeloid-related protein-8 and -14 (MRP-8, MRP-14), which were also identified in inflammation associated with diseases such as cystic fibrosis, chronic bowel disease, and tumors. (Baumann et al., 2011, p. 8; Fessatou et al., 2005; Striz, Jaresova, Lacha, Sedlacek, & Vitko, 2001, p. 14). Clinically, the term "Calprotectin" was first used in 1990 to describe the antimicrobial activity of S100A8/A9 (Steinbakk et al., 1990). The term "Cal-" refers to its calcium binding ability, and "-protectin" refers to the protective function associated with this binding process.

S100A8/A9 has been found in multiple tissues and it is universal *in vivo*. The most common site that S100A8/A9 accumulates is in the innate immune system (Oliviero et al., 2009). Its expression in myeloblast cells is the most abundant and well characterized. Myeloblasts express S100A8/A9 at an early stage; with the differentiation of cells, the concentration of S100A8/A9 drops in most subtypes, except neutrophils and macrophages, which are similar in the amount of S100A8/A9 stored (Perera et al., 2010; Yui, Nakatani, & Mikami, 2003). As the innate immune cells die off, S100A8/A9 is released into serum. Therefore, S100A8/A9 in serum is universal, and its serum concentration is heavily influenced by the status of the immune system such as during an inflammatory response.



(Galeotti, Kaveri, & Bayry, 2015)

High levels of S100A8/A9 have been observed in the synovial membrane of arthritic joints, and on the digestive epithelial layer in the inflammatory bowel disease

(IBD). S100A8/A9 can also be observed on the secretory epithelial layer of an infiltrated leukocytes (Foell, Wittkowski, Vogl, et al., 2007). Some research has suggested that S100A8/A9 is also observed in chondrocytes, with S100A8/A9 staining positive at the superficial zone of articular cartilage in normal tissue; and lost during the process of cartilage damage (P. L. van Lent et al., 2008). Based on this study, the amount of S100A8/A9 from chondrocytes may directly relate to degeneration.

Most recently, a review published in 2010 summarized the current predicted function of S100A8/A9 in arthritis, and suggested that intracellular S100A8/A9 may suppress the differentiation of innate immune cells, while extracellular S100A8/A9 was released by neutrophil necrosis and shown to induce leukocyte migration, suppress matrix metalloproteinase (MMP), anti-oxidant, and calcification (Perera et al., 2010). Therefore, S100A8/A9 may have a role throughout the process of arthritis.

Obesity and high BMI are also correlated to an elevated concentration of serum S100A8/A9 regardless of age and gender (Mortensen et al., 2009), and this elevation is associated with the degree of the other inflammatory biomarkers (Catalán et al., 2011)(Kopeć-Mędrek, Widuchowska, & Kucharz, 2016). An acute bout of exercise can also induce an immediate increase in S100A8/A9 concentration after the exercise bout was complete, but concentration return to baseline values by 24 hours post-exercise. In comparison, the CRP level remained constant within the first 3 hours after exercise (Mooren et al., 2006).

2.4 S100A8/A9 Concentrations

In addition to the antimicrobial function of S100A/A9 in abscess, its inflammatory related pleiotropically function has also been illustrated on the other types of tissues and cells (Steinbakk et al., 1990; Stríz & Trebichavský, 2004). It is worthwhile noting that S100A8/A9 plays a biomolecular role in multiple inflammation pathways, and it is involved in the negative-feedback regulation of inflammation. To be more specific, the concentration of S100A8/A9 determines the trend of proliferation or apoptosis of the cells (Ghavami et al., 2009).

In human serum, the normal range of concentration of S100A8/A9 is 0.3-0.8 mg/L (Johne et al., 1997). This can broaden to 0 and 1.6 mg/L in OA patients with a peak value reported as 4.7 mg/L (Tydén et al., 2013). This fluctuation may be related to the activation of inflammation in the knee joint. Compared with other inflammatory tissues, the S100A8/A9 concentration in OA is much lower. For example, in an abscess, S100A8/A9 concentration can be as high as 1,000-20,000 mg/L (Clohessy & Golden, 1995); in patients with RA, a disease comparable to OA, serum S100A8/A9 concentration can be observed between 1 mg/L to 46 mg/L (Baumann et al., 2011; Striz et al., 2001) depending on whether RA is inactive or active. A similar trend was also shown in synovial fluid: S100A8/A9 concentration in OA patients was reported to be 0.2-2 mg/L, while concentrations climbed to between 2-375 mg/L in RA patients (Johne et al., 1997). In several tumor models, exogenous S100A8/A9 levels over 100 mg/L are needed to initiate apoptosis (Yui et al., 2003). On the other hand, a low S100A8/A9 concentration (10 mg/L) may promote cell growth (Ghavami et al., 2009). Interestingly, both of these concentrations are higher than the peak values observed in OA, suggesting S100A8/A9 in knee OA may not hold a strong biological function. Therefore, serum

S100A8/A9 concentration in knee OA could be mostly related to the onset of inflammation and damaged cartilage.

	S100A8/A9 concentration (mg/L)		
	Serum	Synovial fluid	Peripheral tissue
OA	0 – 1.6 (4.7)	0.2 - 2	
RA	1 - 46	2 - 375	
Abscess			1,000 – 20,000
Tumors	Peripheral concentration: < 10 mg/L: promote cell growth, > 100 mg/L: apoptosis		

However, previous studies have demonstrated that the correlation between S100A8/A9 concentration and the progression of OA is not linear (Zreiqat et al., 2010). Observed in both mice and human, the serum concentration of S100A8/A9 is elevated in the early stage of the disease but dropped later below the control baseline (R. F. Schelbergen et al., 2014; Zreiqat et al., 2010). It is possible that the metabolism of articular cartilage also plays a role in maintaining the normal serum concentration of S100A8/A9. Furthermore, chondrocytes which are not damaged by cartilage degeneration may release S100A8/A9 and influence its baseline, while inflammation contributes to the elevation of serum S100A8/A9 concentration.

Macrophages and neutrophils activated in inflammation are the main source of S100A8/A9. After intraperitoneal injection of heat-killed bacteria into the joint space of

Wistar rats, a migration of neutrophils and elevation of S100A8/A9 concentration was observed simultaneously in the first two days. The amount of macrophages also increased, and reached almost a 4-folds increase above normal even after a drop in S100A8/A9. Interestingly, at day 5, when the macrophage population started to decrease, S100A8/A9 levels were found to increase again. At the end of experiment, the concentration of S100A8/A9 was as high as in day 2. The reason behind the secondary elevation of S100A8/A9 was unclear (Zreiqat et al., 2010, p. 8).

Currently, there is little long-term evidence about how changes in serum S100A8/A9 level influence in knee OA. Previous animal studies stopped before 16 weeks,; and no clinical studies have evaluated the level of serum S100A8/A9 beyond 6 months (Mahler et al., 2015; Zreiqat et al., 2010). Therefore, the response pattern of S100A8/A9 to long term knee OA progression is unclear.

2.5 Comparison between OA and Inflammatory Arthritis

Although inflammation happens in a number of diseases that have different pathogenesis, there is one common feature in all: accumulation of neutrophils (Foell et al., 2004; Foell, Wittkowski, Vogl, et al., 2007). S100A8/A9 concentrations in these diseases are also positively correlated to the disease progression, which are usually affected by the level of inflammation.

RA is the most common arthritis related to autoimmunity. RA is characterized by chronic inflammation and pain of the joints. The hyperactive innate immune system causes synovium inflammation which leads to the destruction of cartilage and joint deformation. Type-B cells in the synovium are the major cell type that is activated and

triggered by the inflammation (Perera et al., 2010). Multiple joints are involved in RA, as the disease progresses. The inflammation caused by RA predominantly involves the synovial membrane, leading to swollen, painful, and stiff joints. To diagnose RA, imaging and blood tests are usually used. X-rays, MRI, and ultrasound are also used to examine the affected joints (Baillet, 2010, p. 100). Rheumatoid factor (RF) is the unique biomarker in RA. Blood tests for RF and anti-citrullinated protein antibodies are critical in distinguishing RA from other arthritic diseases. Treatments of RA focus on reducing inflammation and the consequences of a hyperactive immune system, and utilize drugs including disease-modifying anti-rheumatic drugs and NSAIDs (Weissmann & Korchak, 1984). Compared to RA, the pathological site of OA mainly concentrates on the load bearing area of articular cartilage, generally with a milder inflammation in the synovial membrane. RA starts with inflammation of the synovial membrane and affects neighboring cartilage due to hyperplasia of connective tissues (Manolakis, Kapsoritakis, Tiaka, & Potamianos, 2011).

Although OA and RA show similar pathological changes, the concentrations of S100A8/A9 in the serum and synovial fluid are different in these two diseases. A slight elevation of S100A8/A9 concentration can be found in early stage of OA, but it drops with the progression of OA, and can become even lower than values associated with normal joints (Zreiqat, Howlett, Gronthos, Hume, & Geczy, 2007). While in RA patients, the level of S100A8/A9 can be depressed along with inflammation, but never reseeds back to normal. The trend of S100A8/A9 might indicate that inflammation plays different roles in these two diseases, although inflammation can be observed in both types of arthritis (Heilmann, Wright, Lanerie, Suchodolski, & Steiner, 2014; Mariani et al., 2015;

Meuwis et al., 2013). Furthermore, both OA and RA show destruction of cartilage and inflammation in synovial membrane (Hansson, Eriksson, & Alenius, 2014), but neutrophil-mediated inflammation in synovium is found to be more serious in RA. Similarly, the level of S100A8/A9 in RA is 10-fold higher than in OA (Yui et al., 2003). This may highly suggest that the different levels of S100A8/A9 in associated with RA and OA are directed influenced by from neutrophils.

Additionally, MIA (miscellaneous inflammatory arthritis) is another type of arthritis described as an inflammation of the joint capsule with or without damage to the articular cartilage (Baillet, 2010; Baillet et al., 2010). A study published in 2010 showed that the levels of S100A8 and S100A9 were correlated with the neutrophils in MIA synovial fluid; in contrast, the amount of S100A8 and S100A9 associated with RA is highly elevated compared with the count of neutrophils (Baillet, 2010; Baillet et al., 2010). Since inflammation of synovium exists, it may suggest that cartilage could play a role in producing S100A8 and S100A9.

2.6 Diagnosis of Knee OA

2.6.1 X-ray

X-ray imaging is the most common method of diagnosis for knee OA. The femur and tibia show high-light regions on the image, and the cartilage and peripheral tissues are invisible. As a two-dimensional morphological examination, an anteroposterior weight-bearing knee X-ray is used to evaluate the degree of knee OA progression. The radiology technicians need to adjust the parameters to clearly show the edge of bones and inner-structure without noise. It is important to note that this method of diagnosis does

not show cartilage directly; instead the height of the cartilage is estimated from the joint gap (Leach, Gregg, & Siber, 1970).

The Kellgren & Lawrence (K&L) grading system based on radiographic examination is the most commonly used grading system for knee OA, and it was first published in 1957 (Kellgren & Lawrence, 1957). It categorizes OA into 4 grades, based on the joint space narrowing (to estimate the damage of cartilage), the number of osteophyte, and the presence of sclerosis. The normal joint space should be approximate 1/4 inch (6.4 mm) and 1/8 inch (3.2 mm) on tibia and femur respectively (Anas et al., 2013). Grade 0 indicates a healthy joint with no degenerative changes; Grade 1 is stated as "possible osteophyte formation and joint space narrowing"; Grade 2 presents distinct osteophyte formation; Grade 3 demonstrates "definite joint space narrowing and possible bony deformity"; and Grade 4 shows marked joint space narrowing, severe sclerosis and definite bony deformity. Once joint space narrowing is confirmed, the patient will be graded as 2 or above. Although the K&L grading system is the gold standard of diagnosis and can be used to reflect changes in cartilages, it is not a continuous indicator to guide precise treatment or disease progression. Nevertheless, the K&L grading system still provides the principal evidence for physicians to make an OA diagnosis.

2.6.2 KOOS

The KOOS questionnaire is designed as a scale to access subjective opinions about the respondents' knee signs and symptoms. It was designed by the Institute of Sports Science and Clinical Biomechanics at the University of Southern Denmark, and has been widely used in both research and clinic with relatively good consistency and

compliance (Natalie J. Collins, Misra, Felson, Crossley, & Roos, 2011). It consists of 42 questions within five subscales evaluating five aspects of a patient's OA condition, including pain, symptom, activities of daily living, sport and recreation function, and knee-related quality of life (N. J. Collins et al., 2016). Each question uses five options from 0-4 (None to Extreme) to quantify the severity of the problem. Then, each subscale is calculated by the sum of the answers and transferred to a 0-100 scale. The formula used is: $Subscore = 100 - \frac{Mean\ Score \times 100}{4}$. Although an aggregate total score can be calculated, it is not validated and recommended. The minimal detectable changes for KOOS scoring is between 13-21 at each subscale (Pain: 13.4, Symptoms: 15.5, ADL: 15.4, Sport/Rec: 19.6, and QOL: 21.1) (N. J. Collins et al., 2016; Natalie J. Collins et al., 2011). The higher each sub-score is, the more severe the signs and symptoms of OA. The questionnaire can be repeated weekly, and is designed to evaluate patient pain symptoms and function over the previous 7 days period. KOOS has been proven as a valid and reliable method to evaluate the disability of knee OA patients (N. J. Collins et al., 2016; Ewa M. Roos & Lohmander, 2003). However, its results are based on subjective ratings by the patient, and thus an individual's tolerance can have a great impact on the resultant score. Furthermore, although KOOS can be examined weekly, the relative large minimal-detectable-changes may affect the accuracy of results.

2.6.3 Visual Analogue Scale

The visual analogue scale (VAS) is a measuring instrument based on psychometric response. As pain is a subjective sense, VAS is a practical method for evaluating this outcome. VAS has provided a tool for evaluating pain starting in 1974

(Huskisson, 1982). Usually, VAS is a scale rating from 0 – 10, the greater the number, the worse the pain. Compared with other questionnaires such as KOOS, VAS is more robust in evaluating a patient's immediate or acute pain (Lequesne & Maheu, 2003) with a minimum significant change of 0.9 – 1.1 mm (Kelly, 1998). In general, less than 3% of patients have difficulty using this measurement, but reliability is debatable, especially when used for long-term evaluation (Carr, 2002).

2.6.4 Quantitative Diagnose Method: S100A8/A9

Currently, there is no gold standard for objectively evaluating knee OA disease progression. While the K&L classification shows good clinical reliability, its ability to detect small changes in disease progression or pathogenesis is very limited (Hayashi, Roemer, Jarraya, & Guermazi, 2017). Similarly, the KOOS questionnaire only provides subjective information about disease progression, and offers little objectivity. The ability to objectively quantify small changes in disease progression and pathogenesis using inflammatory biomarkers may significantly enhance current methods used to accurately detect changes in joint health and track disease progression.

S100A8/A9 could be an ideal biomarker to use in order to track changes in joint inflammation associated with knee OA disease progression (R. F. P. Schelbergen et al., 2016). It exists in the chondrocytes and macrophages and could be directly related to the inflammation state of the knee joint. To be more specific, S100A8/A9 is produced from three sources: 1. damaged chondrocytes (Ghavami et al., 2009); 2. remaining healthy chondrocytes (P. L. van Lent et al., 2008); and 3. inflammation activated macrophages (R. F. Schelbergen et al., 2014). As knee OA progresses, the production ratio between these

three sources change, and the concentration of S100A8/A9 may fluctuate. This fluctuation might be a useful tool for tracking the progression of knee OA.

2.7 Treatments of Knee OA

2.7.1 Physical Therapy & Exercise

Excessive loading of the knee joint is thought to trigger inflammation and cartilage damage. Moderate exercise has been suggested as a strategy to improve knee OA (Peeler et al., 2015). Remediated exercise increases the stability of knee joint through improving related muscle strength, such as in quadriceps (Hurley, 1999). Recent studies suggested that the medial gluteal muscle is also likely to have a greater influence on knee stability than quadriceps, because the knee joint needs to compensate for the instability caused by weakened muscle (Steinberg et al., 2017). Physical therapy and exercise have both been successfully used to reduce joint symptoms, but neither form of intervention has been shown structural improved joint health (Knoop et al., 2014; Sharma, 2016).

2.7.2 Weight Loss

Weight loss is necessary for overweight knee OA patient. Obesity has been correlated to the development and severity of knee OA (Arden & Nevitt, 2006; Y. Zhang & Jordan, 2008). Increased cytokines and excessive knee joint loading are both associated with being overweight (Belluzzi et al., 2017). In a recent systemic review on OA, it was concluded that obesity and the local infra-patellar fat pad both engaged in the process of inflammation, and catabolic enzyme productions resulted in knee joint damage

(Belluzzi et al., 2017). Previous studies have also shown that inflammation is induced by excess adipose tissues, and the immune system is also more active than normal (Belluzzi et al., 2017). As the knee joint may need to withstand at least 3 times the body weight during weight bearing activities (Kutzner et al., 2017), the excessive body weight can result in destructive compression of the articular cartilage. Over time, the accumulation of large these large compression forces exceeds the repair threshold of the articular cartilage, and knee OA development will ensue.

2.7.3 Total Knee Replacement

Knee joint replacement (total knee arthroplasty-TKA) is a terminal method of treatment provided to patients with severe knee OA (Felson et al., 2000; W. Zhang et al., 2008).

In Canada, about 60,000 patients receive partial or total knee replacement each year. This number has increased 15.7% between 2011 to 2016. Patients suffering from OA made up 97.9% of all knee replacement cases in 2014 – 2015, and it is estimated that this number will continue to climb to 99% in future years (Information (CIHI), 2017). Consistent with the demographics of knee OA, females had a higher rate (61.6% in 2014 - 2015) than males; and the older population (greater than 65 years) were the largest patient population which hold 54.5% in total (“cjrr-annual-report-2016-en.pdf,” n.d.).

2.7.4 LBPP Treadmill

Information on the Lower Body Positive Pressure (LBPP) treadmill (Alter-G Inc – Menlo Park, CA) exercise was first published about metabolism and neural activities of the lower body in the 1980's (Bennett, Tighe, & Wegg, 1982). A few years later, the focus of LBPP shifted to its effect on the cardiovascular system (Nagano et al., 1987). Although some physiological changes were discovered, many had little clinical relevance. At the same time, the LBPP technology drew attention from NASA.

Most recently, as a modern load-control exercise strategy, the LBPP treadmill has been used for studying diseases which are related to over-loading of the lower extremities such as knee OA. Excessive loading at the knee joint was thought to be a potential trigger for inflammation, therefore exercising in an "anti-gravity" setting may help prevent the progression of knee OA. The principle behind this exercise is to decrease apparent weight through increasing air pressure, so the load felt by the knees during slow running is decreased. However, the increased body weight support could alter the lower limb kinematics from a normal gait. To assess the exercise for clinical use, a study published in 2016 investigated body kinematics during LBPP exercise (60 – 80% VO_2 peak velocities). Results suggested that at 20% body weight support by LBPP treadmill partly changed the gait; and 40% body weight support altered normal gait completely. As a conclusion, 20% or less weight support was suggested for clinical application ("Effect of Body-Weight-Support Running on Lower-Limb Biomechanics," 2016). Recent studies have proven that moderate LBPP exercise can significantly improve pain and function of the knee, increase strength of thigh muscle groups, and help to rebuild the patient's self-confidence (Peeler et al., 2015; Takacs et al., 2013). It is thought that moderate loading may help reduce level of inflammation, and the load on knee cartilage. A study

demonstrated that serum COMP in healthy participants walking on + 40% body weight was 14% higher than healthy participants walking on - 40% body weight (Denning, Winward, Pardo, Hopkins, & Seeley, 2015). Although no research has looked at serum S100A8/A9 concentration under LBPP exercise, it is possible that serum S100A8/A9 concentration will be significantly decreased because of diminished activity in leukocytes and less damage to chondrocytes.

2.7.5 Creatine Supplementation

A non-essential nutrient, creatine ($C_4H_9N_3O_2$) is a nitrogenous organic acid which assists in the formation of adenosine triphosphates (ATP) (Pinto, Botelho, Pimentel, Campos-Ferraz, & Mota, 2016). *In vivo*, it is produced in the kidney and liver, and transferred to skeletal muscles by blood for storage (95% in total) (Twycross-Lewis, Kilduff, Wang, & Pitsiladis, 2016). Creatine can be activated by creatine kinase through phosphorylation, and then works as an energy buffer in muscles. The biological half-life of phosphocreatine is 3 hours. Uptake of creatine is accompanied by water retention, which may increase cardiovascular load and metabolic pressure on the liver and kidney. However, there is still no conclusive evidence to support its side effects in human. Data from the European Food Safety Authority (EFSA) in 2004 recommended 3 grams per day of creatine as the oral long-term risk-free dose (European Food Safety Authority (EFSA), 2004). In later studies, 20 grams of creatine per day was also shown as a safe dose (Bizzarini & De Angelis, 2004).

Creatine supplementation has clearly demonstrated its ability to increase muscle strength (Twycross-Lewis et al., 2016). After active uptake by the muscle cells, creatine

with creatine kinase maintain ATP production through transfer of phosphate group to ADP, and in turn increase muscle endurance and explosive force (Graham & Hatton, 1999). Creatine also helped decrease the generation of reactive oxygen species, which is a trigger for inflammation and apoptosis (Kitzenberg et al., 2016).

One of the main functions of creatine is to reduce the level of inflammation. It remains uncharacterized in the human body (Kitzenberg et al., 2016) and has been observed under multiple scenarios, including inflammatory bowel diseases, ischemic injuries, and thromboembolic disorders related to the cardiovascular system (Kitzenberg et al., 2016; Nomura et al., 2003).

The anti-inflammation function of creatine appears after acute exercises (Bassit, Curi, & Costa Rosa, 2008, p. 2; Deminice, Rosa, Franco, Jordao, & de Freitas, 2013; Santos, Bassit, Caperuto, & Costa Rosa, 2004). In previous studies, the intake of creatine did not change the inflammatory level before exercise, but significantly depressed the increasing trend of inflammatory biomarkers (such as CRP, TNF- α, and ILs) after exercise, from one hour to forty-eight hours (Bassit et al., 2008, p. 2; Santos et al., 2004); whereas the oxidative stress markers (anti-oxidant enzymes) were not influenced by creatine supplementation (Deminice et al., 2013).

As a health product, its benefits for knee OA patients remain unclear. One study in 2011 illustrated that a 12-week creatine supplementation helped to significantly improve the knee OA disability and functions (Neves et al., 2011). However, in a recent study with the same creatine intake strategy, the functional improvements were not demonstrated, neither did the depression of inflammatory biomarkers occur (Cornish & Peeler, 2018). Its molecular mechanism as a health product remains elusive.

3. HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

At present, knee OA is still an incurable disease with unclear pathophysiology and progression. Symptomatic treatments can only postpone the need for eventual joint replacement surgery. Many have undertaken research to study the disease and find other treatment options, but progress is slow. One critical reason is that there is still no effective and objective diagnostic method.

As we have discussed above, S100A8/A9 is relatively specific to cartilage damage and peripheral inflammation. Therefore, the **Central Hypothesis** is that**:** the serum S100A8/A9 level is correlated to progressive inflammation in knee OA, and the serum S100A8/A9 concentration can be used to track OA disease progression and examine the effectiveness of knee OA treatments such as LBPP exercise and Creatine supplementation.

3.2 Objectives

Our primary objectives will focus on the inflammatory serum S100A8/A9 biomarker, including its concentration in knee OA patients, its correlations with current evaluation methods (KOOS), and if there is any difference between LBPP exercise and Creatine supplementation.

Objective No. 1: Determine the concentration of serum S100A8/A9 in individuals diagnosed with mild to moderate knee OA.

Objective No. 2: Determine whether serum S100A8/A9 levels in individuals diagnosed with mild to moderate knee OA are affected by a 12-week low-load walking exercise intervention or dietary supplementation using creatine monohydrate.

Objective No. 3: Determine whether a relationship exists between KOOS scoring and serum S100A8/A9 levels in individuals diagnosed with mild to moderate knee OA.

We also set additional objectives for serum COMP, which is less related to inflammation and more to mechanical stress and degradation of articular cartilage. In addition to serum S100A8/A9, we will also check if serum COMP concentration corresponds to KOOS.

Objective No. 4: Determine whether serum COMP levels in individuals diagnosed with mild to moderate knee OA are affected by a 12-week low-load walking exercise intervention or dietary supplementation using creatine monohydrate.

Objective No. 5: Determine whether a relationship exists between KOOS scoring and serum COMP levels in individuals diagnosed with mild to moderate knee OA.

4. MATERIALS AND METHODS

4.1 Participants Population

Participants were recruited from the patient population at Pan Am Clinic. The inclusion criteria included: 1. 45-65 years of age; 2. Body mass index (BMI) over 25 kg/m^2; 3. Knee OA in K&L grades II or III (mild to moderate) in one or both knees; 4. Knee pain during normal daily activities. In addition, patients exclusion criteria were: 1. Severe knee OA diagnosed by radiographic examination; 2. History of recent trauma or operation in the lower extremities; 3. History of immobilization in the lower extremities;

4. Failure to provide research-related responses due to language barrier or mental illness; 5. History of diseases including but not limited to CBD, SLE, Ankylosing Spondylitis, RA, chronic reactive arthritis, and diseases affecting the innate immune system or metabolic system; or 6. Failure to attend follow-up sessions. Each participant was assigned a code at the beginning of the study to ensure anonymity. This study was single blinded where the participants did not know what intervention they attended. This clinical trial was approved by University Research Ethics Board.

4.2 Baseline Evaluation

Baseline testing was performed on participants on the following parameters: 1. Body anthropometry, 2. KOOS questionnaire, 3. Radiographic evaluation of knee joints using K&L classification, 4. Blood draw sampling for biomarker, and 5. Pain VAS during full weight bearing (FWB) treadmill walking for 30 minutes at 3.1 mph with 0% incline.

4.2.1 KOOS Questionnaire

The KOOS questionnaire is a common measurement used to trace the symptomatic progression of knee OA in clinical practice. The minimum test period could be as short as one week (E. M. Roos, Roos, Lohmander, Ekdahl, & Beynnon, 1998; Ewa M. Roos & Lohmander, 2003). The KOOS questionnaire has 42 items in total, which are categorized into five subscales named: symptoms (7 items), pain (9 items), activities of daily living (ADL) function (17 items), sports and recreation function (5 items) and quality of life (4 items). The participants had five possible answer options on a scale of 0

– 4, with 0 presenting none to 4 presenting extreme. For any in-between-number answer by participants, the more severe option were chosen instead as the final answer (E. M. Roos et al., 1998). Each subscale was calculated independently. The final scores were transformed to a scale ranged from 0 to 100, with 0 indicating severe knee problems and 100 indicating a normal knee status (Iwamoto, Sato, Takeda, & Matsumoto, 2011).

A copy has been attached in the appendix of this thesis (Appendix 8.2).

4.2.2 Kellgren & Lawrence Grade

Copies of the X-ray report of each participant were collected from their physicians. "Osteophyte" and "Joint space narrowing" in the report were used as key words to re-evaluate the severity of a participant's self-assessed knee condition. Following the K&L grading system, the participants were labeled into Grade II or Grade III (Kellgren & Lawrence, 1957).

4.2.3 Anthropometric Measurement

Anthropometric measurements of all participants were collected at the Pan Am Clinic (Winnipeg Regional Health Authority's "Centre of Excellence for Musculoskeletal Injury Assessment and Treatment") using the same devices. Height (meters) of the participants was measured at peak inhalation and without shoes. Weight (kilograms) was also determined to calculate the BMI (kg/m^2).

4.2.4 Blood Sample Analysis

Blood samples were collected and processed by technicians at the Pan Am clinic. Twenty (20) mL blood was drawn from the antecubital vein by venipuncture under sterile conditions. Blood samples were centrifuged at 1065g for 15 minutes. Approximately 8 mL serum was collected after centrifugation. Serum was aliquoted into 1 mL Eppendorf tubes before being transported back to the hematological laboratory in the faculty of Kinesiology and Recreation Management at the University of Manitoba. Samples were stored in -80°C freezer (Thermo Fisher Scientific (Asheville) LLC, United States) until analysis.

4.2.5 ELISA

Hematological analyses were performed on baseline and follow-up blood samples from all participants. ELISA kits are produced by R&D System (Inc. 614 McKinley Place NE Minneapolis, USA).

Seven biomarkers were examined using ELISA according to the manufacturer's protocol: S100A8/A9, C-reactive protein, interleukin-1β, interleukin-6, interleukin-10, tumor necrosis factor-α, and cartilage oligomeric matrix protein. The microplate was read by the Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., USA). Raw data were collected by the Gen5 software and the final concentrations of biomarkers were calculated. All assays were analyzed in triplicates on the same microplate.

4.2.6 Pain VAS during Full Weight Bearing Exercise

All participants walked on the LBPP treadmill at 0% body-weight-support for 30 minutes. Before the exercise began, participants wore a pair of neoprene shorts to create

an airtight seal with the treadmill air chamber. During the expansion of the air chamber, the participants were asked to remain full foot-ground contact, which allowed the treadmill to correctly calculate their body weight. A 1 – 10 VAS scale was placed in front of the participants to rate knee pain every 5 minutes; and a waist pedometer was put on to read the heart rate during walking.

The first 5 minutes in the session was spent as a warm-up session, with the walking speed gradually increased over time from 1.6 mph to the target speed, 3.1 mph (Browning & Kram, 2007). At this speed, the participants are willing to reach their target HR zone (65 – 85% of their max HR). For the next 25 minutes, pain VAS scores were recorded every 5 minutes (at 5, 10, 15, 20, 25, and 30 minutes). The treadmill display screen was covered to prevent participants viewing.

4.3 Study Design

Following completion of baseline assessment, participants were randomly assigned one of three groups.

4.3.1 Exercise Group

Participants walked at 3.1 mph at 0^0 incline for 30 minutes, 3 times per week on an Alter-G treadmill using LBPP support to minimize load on the lower extremities. The air chamber pressure will be adjusted to lift participants and relieve pressure on the lower body. At the start of the initial 5-minute warm-up session, participants were put on a load at 80% of body weight, and it was adjusted to a load right before discomfort of the knee appeared in each individual. Participants were not told the level of LBPP support

received. This protocol was designed and applied in a previous study from our laboratory (Peeler et al., 2015).

4.3.2 Creatine Group

The selected creatine monohydrate dose was based on the reported maximum safe dose (Bizzarini & De Angelis, 2004), which was employed in a previous study from Brazil (Neves et al., 2011). Participants were supplemented with 5 grams of creatine monohydrate 4 times a day in the first week; thereafter, 5 grams were supplemented per day as a maintenance dose for the remaining 11 weeks.

4.3.3 Control Group

Similar to the creatine group, participants were supplemented with 5 grams of a placebo (maltodextrin) 4 times per day in the first week, and 5 grams per day as a maintenance dose for the remaining 11 weeks.

4.4 Follow-up Evaluation

Following the 12-week intervention, the same tests were performed as the baseline evaluations.

4.5 Statistical Analysis

Statistical analysis was completed using SAS (Copyright © SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513, USA). The statistical methods used in this study included description, longitudinal analysis, correlation analysis, and linear

regression. At first, all of the outcomes were categorized in groups and shown in tables as descriptive statistics (including mean ± standard deviation). Then, a longitudinal analysis was performed to test the dependent variables, including the parametric data (ROM, KOOS, VAS, S100A8/A9 and COMP concentrations); meanwhile several independent variables were controlled (interactions of time/group, age, gender, BMI, unilateral or bilateral knee OA, and K&L grade). Finally, correlation analysis and linear regression were used to determine whether a relationship existed between biomarkers' levels and KOOS scoring. Significance was set at $p < 0.05$.

Longitudinal multilevel analysis, as well as repeated measurements, is a statistical method used to investigate multiple factors at the same time (King et al., 2017). As more factors are controlled in this model, more levels can be established (West, Ryu, Kwok, & Cham, 2011). The equations of longitudinal analysis in this study were:

Level 1: $y = \beta_{01} \times \text{Group} + \beta_{02} \times \text{Age} + \beta_{02} \times \text{Gender} + \beta_{03} \times \text{Affected} + \beta_{04} \times \text{K\&L} + \beta_{05} \times \text{BMI} + \beta_{1j} \times \text{Time} + \varepsilon$

Level 2: $\beta_{1j} = \gamma_{10} + \gamma \times \text{Group} + \mu_{1j}$

The purpose of longitudinal analysis is to use independent variables to predict the dependent variables (y) (Atkins, 2005; West et al., 2011). The changed amount of dependent variables is determined by the changed amount ($\gamma, \text{coefficiant}$) of independent variables.

Compared with ANOVA (Analysis of variance) and multi-factorial regression, the longitudinal multilevel method of analysis is considered to be more precise (West et al., 2011). It utilizes a combination of ANOVA and multi-factorial regression. It allows level 2 factors (which evaluate the interactions between any of the two factors in level 1) to be

evaluated in a manner that is similar to the principle of ANOVAs (for example in the present study, the level 2 factor of S100A8/A9 concentration was examined in different groups between baseline and follow-up). At the same time, the longitudinal multilevel method of analysis also works as a multi-factorial regression, evaluating the background information of a specific study population to determine if inclusion criteria may impact the outcomes (dependent variables, y), and suggests the possible compounding biases on sampling or grouping. Therefore, the longitudinal multilevel model of analysis not only can be used to evaluate the effectiveness of specific interventions, but also can provide the information about potential bias.

In our study, the controlled independent variables included: Time (baseline and follow-up), Group (LBPP, Creatine, Control), the Interaction between Time and Group, Age, Gender (male and female), BMI, Affected (unilateral and bilateral), and K&L grade (Grade II and Grade III). The interactions between Time and Group would demonstrate that if the interventions had a treatment effect, after considering the influence of time and groups; the other independent variables (Time, Age, Gender, BMI, Affected, and K&L) would show differences between the mild and moderate knee OA populations, which had excluded the effects of interventions.

In order to simplify the description of the results from the longitudinal analysis, the significant ($p < 0.05$) and marginal significant ($p < 0.10$) results for each section were presented in the following chart:

Summary of the Significance in Longitudinal Analysis

Dependent Variables	Independent Variables	Amount of Change	P value
y: (ROM, VAS, KOOS, or Biomarkers)	Time (Follow-up – Baseline; All samples)		
	Group (LBPP, Creatine, or Control; no time effect)		
	Time*Group (Follow-up – Baseline; LBPP, Creatine, or Control)		
	Age (per 1 year older)		
	Gender (Male - Female)		
	BMI (per 1 kg/m^2 increased)		
	Affected (Unilateral - Bilateral)		
	K&L (Grade II – Grade III)		

The correlation analysis and Pearson linear correlations between the outcomes were demonstrated. The value of r, which presents the correlation coefficient, measures the strength of the relationship; and the positive or negative sign shows the direction of the relationship. Based on a publication in 2012 (Mukaka, 2012), the level of the strength of correlation coefficient is listed as below:

Size of Correlation	Interpretation
.90 to 1.00 (−.90 to −1.00)	Very high positive (negative) correlation
.70 to .90 (−.70 to −.90)	High positive (negative) correlation
.50 to .70 (−.50 to −.70)	Moderate positive (negative) correlation
.30 to .50 (−.30 to −.50)	Low positive (negative) correlation
.00 to .30 (.00 to −.30)	negligible correlation

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3576830/table/T1/

In this section, the correlations between the independent variables (KOOS, VAS, biomarkers) were tested. We alternated the scores and concentrations to the "change ratio $[\frac{Followup - Baseline}{Baseline}]$, and examined its correlation, which was able to present if theirs trends corresponded to each other's.

Only significant ($p < 0.05$) and marginal significant ($p < 0.10$) linear correlations were shown.

5. RESULTS

5.1 Patient Population and Demographic Information (Table 1)

Twenty-seven participants were recruited into this study. One subject failed to meet inclusion criteria and was excluded because of a lack of pain during daily living. In total, twenty-six participants were enrolled in the 12-week interventions. Over the course

of the study, two participants withdrew from participation due to pain in the knee joint and failure to follow-up. During the final analysis, one outlier was found based on the serum S100A8/A9 concentration (Figure 1). Therefore, a total of four cases were excluded before the final statistical analysis, and the final sample size was twenty-three.

The demographics of the participants are summarized in Table 1a and 1b. The average age in each group was between 56.7 to 58.8 years. The average duration of knee OA symptoms ranged from 9.2 to 14.5 years. Twelve participants were radiographically diagnosed to be in the K&L grade II category, while the other eleven were in grade III. Twelve participants had unilateral knee OA and the other eleven had bilateral. Eleven participants reported a previous long-term knee injury. Currently, all of the participants suffered from pain in daily living, with moderate functional difficulties. Besides the pain, six of them reported observable swelling in the affected knee joint.

5.2 Description and Longitudinal Analysis

5.2.1 Anthropometric Measurements (Table 2)

The anthropometric measurements including weight, height, and BMI were shown in Table 2. BMI greater than 25 kg/m^2 was defined as being over-weight. After the 12-week intervention, the change in BMI in each group was 0.54 (LBPP), 0.2 (Creatine), and -0.39 (Control). As expected, the LBPP exercise and the Creatine supplementation maintained body weight, as well as BMI.

5.2.2 Joint Range of Motion in the Affected Knee (Table 3)

Range of motion in the joint during flexion and extension of the lower extremities (hip, knee, and ankle) is presented in Table 3. The number of cases was calculated based on the following parameters: 1. in unilaterally affected participants, the affected knees counts as one case; 2. in bilaterally affected participants, each leg is counted independently. Thus, the case number in ROM examinations was different for each analyses.

In the LBPP exercise group, a total of nine OA affected extremities were considered. The average movement of hip flexion showed a 13.92° decrease after 12-week of exercise. No significant change was found in Creatine supplementation group (n = 14). In the control group (n = 11), the average knee flexion increased by 5.12° during follow-up.

Generally, the average degrees of movements in the hip, knee, and ankle on sagittal plane (Hip Flexion 78-83°, Extension 9.3-10.2°; Knee Flexion 109.7-114.7°, Extension 0.6-1.3°; Ankle Plantar flexion 37.3-37.8°, Dorsi flexion 10.7-14.6°) were limited compared to normal ranges of motion in a healthy population (Hip Flexion 90–135°, Extension 10-30°; Knee Flexion 130–140°, Extension 5–10°; Ankle Plantar flexion 40–50°, Dorsi flexion 15–20°).

In the longitudinal analysis, only one parameter showed a significant change, both statistically and clinically, hip flexion: the ROM in males was -13.8° less than the ROM in females ($p = 0.0335$). There was a marginal significance ($p = 0.0513$) found in the Creatine group: the 12-week creatine supplementation had lower ankle dorsi-flexion by -3.77°. In addition, age, gender, BMI, and K&L grade had little impact on the movement of lower extremities.

5.2.3 VAS Scoring for FWB walking session (Table 4)

Table 4 presents the VAS scores for a self-reported knee pain during full weight bearing walking session on the treadmill.

All of the groups reported positive results at follow-up. The participants in both LBPP and Creatine groups reported greater reduction in pain than the Control group after the 12-week intervention. In the LBPP group, the average VAS score reduced from 2.09 to 0.72 (a difference of 1.37); and in the Creatine group, the score reduced from 1.62 to 0.70 (a difference of 0.92). In the control group, the improvement of VAS pain was 0.71.

However, the result in longitudinal analysis suggested that the interventions did not affect VAS score. Marginal significances were observed in gender, number of affected knees, K&L grade, and BMI. Overall, males reported less pain than females (-0.95). To our surprise, participants with one-leg knee OA or those that experienced milder knee OA, suffered more severe pain (0.97 and 0.91).

5.2.4 KOOS Scores (Table 5)

The KOOS scoring is one of the most important outcomes in this study. This questionnaire is a subjective measurement of knee OA symptoms that is broken down into 5 categories: knee OA related symptoms, pain, activity of daily living, sport and recreation function, and quality of life. The results are shown in Table 5.

Most of the outcomes demonstrated a difference of less than 6 points. According to the suggested minimal detectable changes, these differences were not clinically significant (N. J. Collins et al., 2016). Additionally, for LBPP exercise group, the average

score of Sport/Rec subscale exhibited the greatest change from 37 to 55; In the Creatine group, the score of QOL increased from 38 to 50.

These two changes were supported by longitudinal analysis. The 12-week creatine supplementation improved the score of QOL by 12.5 ($p = 0.0467$). The LBPP exercise improved sport/recreation function by 17.7 ($p = 0.0533$). It was found that males had better sport/recreation improvement than females. Similar to the results from VAS scoring, participants with more severe knee OA (K&L Grade III) showed better functions than Grade II patients, including activity of daily living function, sport/recreation function, and quality of life.

5.2.5 Bloodwork (Table 6)

Table 6 provides a summary of the serology results. Six biomarkers were tested in the laboratory by ELISA using high-sensitive kits. Concentrations of serum S100A8/A9 and serum COMP were tested along with other inflammatory biomarkers including CRP, IL-1β, IL-6, and TNF-α. Overall, most biomarkers remained within normal ranges after 12-weeks, with the exception being that CRP concentration was slightly increased.

The average concentrations of serum S100A8/A9 in three groups were between 285 to 487 μg/L, which is slightly higher than the normal average value (300 μg/L). The concentration of CRP, IL-1β, IL-6, and TNF-α were all within normal ranges. The average serum concentration of COMP was slightly higher than would be observed in a healthy population, which was consistent with the findings of previous knee OA investigation (Wisłowska & Jabłońska, 2005)

In the longitudinal analysis, S100A8/A9 positively correlated with BMI (p = 0.0144). With a 1 kg/m^2 increased in BMI, there was on a 17.34 μg/L increase in S100A8/A9 serum level. COMP concentrations elevated 24.27 μg/L within 12 weeks (p = 0.0457), but our interventions did not influence this trend.

5.3 Correlations

5.3.1 Change ratio correlations between S100A8/A9 and KOOS

Consistent with the primary objectives of our study, the Figure 2 illustrated the relationships about the change ratios of serum S100A8/A9 concentration and KOOS.

Marginally significant linear correlations were demonstrated between S100A8/A9 & KOOS pain (p = 0.0865), and S100A8/A9 & KOOS activity of daily living (p = 0.0902). The coefficients were 0.365 and 0.361 respectively, which illustrated low-positive correlations. These might suggest that the more S100A8/A9 elevates, the lower the knee pain and the higher the function noticed by knee OA patients.

5.3.2 Change ratio correlations between COMP and KOOS

Figure 3 illustrates the relationships between changes in ratios of serum COMP and KOOS scoring.

Three negative correlations were demonstrated. The change ratio correlationsbetween COMP and KOOS pain/symptoms were weak (r = -0.367 / -0.464, p = 0.0847 / 0.0259), the correlation between COMP and KOOS activities of daily living were moderate and statistically significant (r = -0.608, p = 0.0021). These results

indicated that the more COMP concentration decreased, the more KOOS scores increased (ie. symptom, pain, and daily activity function scores all improved).

6. TABLES AND FIGURES

Table 1a. General Demographic Information - Mean ± SD (range)

Demographic Info	LBPP N = 7	Creatine N = 8	Placebo N = 8
Gender (Male / Female)	2 / 5	4 / 4	5 / 3
Age (years)	58.8 ± 6.8 (45.1 – 66.6)	56.9 ± 8.3 (46.7 – 65.9)	56.7 ± 6.8 (46.8 – 65.9)
Duration of Symptoms (years)	9.2 ± 11.1 (0.5 - 30)	14.5 ± 16.5 (1.5 - 48)	13.7 ± 10.9 (1 - 31)
Kellgren and Lawrence Grades (Grade II / III)	6 / 1	3 / 5	3 / 5
Affected Knee (unilateral / bilateral)	5 / 2	2 / 6	5 / 3

The general demographics illustrate a participant population with older and mild to moderate knee osteoarthritis.

Table 1b. Knee OA Details Information - Mean ± SD (range)

Demographic Info	LBPP N = 7	Creatine N = 8	Placebo N = 8
Pain VAS (0 - 10)	6.0 ± 1.8 (4 - 8)	4.6 ± 2.7 (1 - 10)	5.8 ± 3.1 (2.5 - 10)
Function VAS (0 - 10)	5.8 ± 2.4 (1 – 7.5)	6.6 ± 2.0 (4 - 10)	5.4 ± 2.2 (1 - 8)
Leg Alignment (Varus / Normal / Valgus)	1 / 6 / 0	0 / 5 / 3	0 / 8 / 0
Long Term Injury (Yes / No)	3 / 4	2 / 6	6 / 2
Swelling (Yes / No)	1 / 6	4 / 4	1 / 7
SF-12 PCS	40.99 ± 5.62 (32.7 – 48.0)	43.53 ± 8.65 (29.2 - 53)	40.95 ± 9.85 (26.8 – 54.7)
SF-12 MCS	52.56 ± 11.23 (36.0 – 65.7)	52.01 ± 13.09 (23.1 – 65.2)	48.50 ± 9.97 (31.9 – 60.7)

Physical Component Summary; PCS
Mental Component summary; MCS

The knee OA demographics illustrate a participant population with pain and function disorders in general.

Table 2. Anthropometric Description - Mean ± SD (range)

Anthropometry	LBPP N = 7		Creatine N = 8		Placebo N = 8	
	Baseline	Follow-Up	Baseline	Follow-Up	Baseline	Follow-Up
Weight (kg)	90.93 ± 23.91 (62.02 – 132.5)	89.29 ± 22.95 (61 - 131)	87.56 ± 19.69 (60.5 – 121.5)	88.06 ± 19.58 (61.5 - 122)	100.94 ± 19.86 (80.5 – 145.5)	99.81 ± 19.68 (80.5 – 143.5)
Height (m)	1.674 ± 0.033 (1.575 – 1.83)		1.719 ± 0.099 (1.59 – 1.85)		1.741 ± 0.093 (1.62 – 1.89)	
BMI (kg/m^2)	32.05 ± 5.53 (25 – 39.6)	31.51 ± 5.47 (24.6 - 39)	29.35 ± 4.29 (23.9 – 35.5)	29.55 ± 4.41 (24.3 – 35.6)	32.63 ± 4.69 (26.6 – 40.7)	32.24 ± 4.51 (26.6 – 40.2)

Table 3. Joint Range of Motion in the Affected Knee Description - Mean ± SD (range)

Joint Movement (degrees)	LBPP N = 9		Creatine N = 14		Placebo N = 11	
	Baseline	Follow-Up	Baseline	Follow-Up	Baseline	Follow-Up
Hip flexion	92.04 ± 15.42 (64 - 110)	78.12 ± 22.83 (43 - 108)	78.21 ± 20.86 (23 - 100)	81.76 ± 21.09 (28 - 102)	79.69 ± 7.59 (65 - 90)	77.88 ± 13.84 (43 - 95)
Hip Extension	9.07 ± 4.87 (3 - 20)	8.13 ± 2.55 (5 - 12)	10.22 ± 3.63 (5 - 18)	11.76 ± 4.15 (5 - 18)	8.17 ± 3.45 (5 - 15)	8.51 ± 4.91 (5 - 22)
Knee Flexion	113.18 ± 12.60 (95 - 135)	118.01 ± 5.76 (105 - 125)	114.74 ± 6.49 (105 - 127)	116.16 ± 4.22 (112 - 125)	108.50 ± 10.27 (95 - 125)	113.62 ± 9.19 (100 - 125)
Knee Extension	0.73 ± 2.20 (0 - 7)	0.56 ± 1.67 (0 - 5)	0.81 ± 1.81 (0 - 5)	0.59 ± 1.54 (0 - 5)	1.51 ± 3.36 (0 – 8)	1.82 ± 3.37 (0 - 10)
Ankle Planter Flexion	38.17 ± 10.74 (23 - 54)	35.22 ± 9.08 (22 - 47)	37.36 ± 6.90 (20 - 48)	37.67 ± 7.98 (22 - 52)	36.20 ± 10.96 (20 - 60)	38.61 ± 4.99 (28 - 45)
Ankle Dorsi Flexion	14.23 ± 4.10 (7 - 18)	15.90 ± 3.91 (10 - 20)	11.52 ± 3.44 (7 - 20)	10.08 ± 3.72 (3 - 17)	10.15 ± 3.98 (5 - 17)	12.57 ± 5.71 (4 - 23)

Table 3a. Joint Range of Motion in the Affected Knee – Summary of the significance in Longitudinal Analysis

Dependent Variables	**Independent Variables**	**Amount of Change**	**P value**
Hip Flexion (degree)	Gender (Male – Female)	-13.8˚	0.0335 *
	BMI (per 1 kg/m^2 increased)	1.1˚	0.0809
Hip Extension (degree)	Age (per 1 year older)	-0.3˚	0.0033 **
Knee Flexion (degree)	Age (per 1 year older)	-0.5˚	0.0108 *
	BMI (per 1 kg/m^2 increased)	-0.9˚	0.0002 **
Knee Extension (degree)	Gender (Male - Female)	0.05˚	0.0376 *
Ankle Dorsi-flexion (degree)	Time*Creatine Group (Follow-up – Baseline, Creatine)	-3.77˚	0.0513
	K&L (Grade II – Grade III)	2.44˚	0.0930
Ankle Plantar-flexion (degree)	BMI (per 1 kg/m^2 increased)	0.6˚	0.0520

*$p < 0.05$, **$p < 0.01$

Table 4. VAS Scoring for FWB Description- Mean ± SD (range)

Groups	LBPP N = 7		Creatine N = 7		Placebo N = 8	
	Baseline	Follow-Up	Baseline	Follow-Up	Baseline	Follow-Up
VAS	2.09 ± 1.19 (0.1 – 3.8)	0.72 ± 0.71 (0 – 2)	1.62 ± 1.20 (0 – 3.8)	0.70 ± 0.85 (0 – 2)	2.88 ± 1.79 (0 – 6.2)	2.17 ± 1.95 (0 – 5.3)

Table 4a. VAS Scoring for FWB – Summary of the Significance in Longitudinal Analysis

Dependent Variables	**Independent Variables**	**Amount of Change**	**P value**
VAS (0-10 points)	Gender (Male – Female)	-0.95	0.0902
	Affected (Unilateral – Bilateral)	0.97	0.0780
	K&L (Grade II – Grade III)	0.91	0.0985
	BMI (per 1 kg/m^2 increased)	0.10	0.0806

*p < 0.05, **p < 0.01

Table 5. KOOS Scores Description - Mean ± SD (range)

Subscale	LBPP N = 7		Creatine N = 8		Placebo N = 8	
	Baseline	Follow-Up	Baseline	Follow-Up	Baseline	Follow-Up
Symptoms	63.64 ± 10.24 (53 - 83)	68.70 ± 9.37 (56 - 78)	64.24 ± 17.41 (33 - 89)	64.93 ± 17.25 (36 - 94)	63.19 ± 17.17 (44 - 89)	61.46 ± 17.09 (36 - 86)
Pain	64.29 ± 11.85 (50 - 82)	70.92 ± 10.99 (54 - 82)	61.16 ± 18.75 (32 - 86)	63.84 ± 13.15 (43 - 82)	63.39 ± 13.60 (46 - 82)	68.30 ± 17.65 (39 - 93)
ADL	68.28 ± 9.14 (56 - 84)	74.58 ± 8.31 (63 - 88)	69.57 ± 16.28 (38 - 88)	72.68 ± 15.69 (54 - 99)	68.38 ± 20.86 (41 - 97)	68.38 ± 22.22 (38 - 94)
Sport/Rec	37.14 ± 14.68 (15 - 60)	55.71 ± 18.58 (25 - 75)	45.00 ± 22.52 (15 - 75)	41.88 ± 27.64 (0 - 80)	48.13 ± 28.90 (10 - 90)	48.13 ± 35.75 (10 - 100)
QOL	47.32 ± 7.95 (38 - 56)	51.79 ± 16.02 (31 - 75)	38.28 ± 20.44 (0 - 63)	50.00 ± 19.19 (13 - 75)	46.09 ± 23.61 (0 - 75)	45.31 ± 21.06 (19 - 75)

ADL – Activities of Daily Living,
Sport/Rec – Sport/Recreation Function,
QOL – Quality of Life

Table 5a. KOOS Scores – Summary of the Significance in Longitudinal Analysis

Dependent Variables	**Independent Variables**	**Amount of Change**	**P value**
Symptom (0 – 100 points)	Gender (Male – Female)	-2.0	0.0587
	K&L (Grade II – Grade III)	-9.8	0.0670
Pain (0 – 100 points)			
Activity of Daily Living (0 – 100 points)	K&L (Grade II – Grade III)	-16.2	0.0071 **
Sport/Recreation Function (0 – 100 points)	Time*LBPP Group (Follow-up – Baseline, LBPP)	17.7	0.0533
	Gender (Male – Female)	20.1	0.0231 *
	K&L (Grade II – Grade III)	-21.5	0.0146 *
Quality of Life (0 – 100 points)	Time*Creatine Group (Follow-up – Baseline, Creatine)	12.5	0.0467 *
	K&L (Grade II – Grade III)	-13.3	0.0473 *

*$p < 0.05$, **$p < 0.01$

Table 6. Bloodwork Description - Mean ± SD (range)

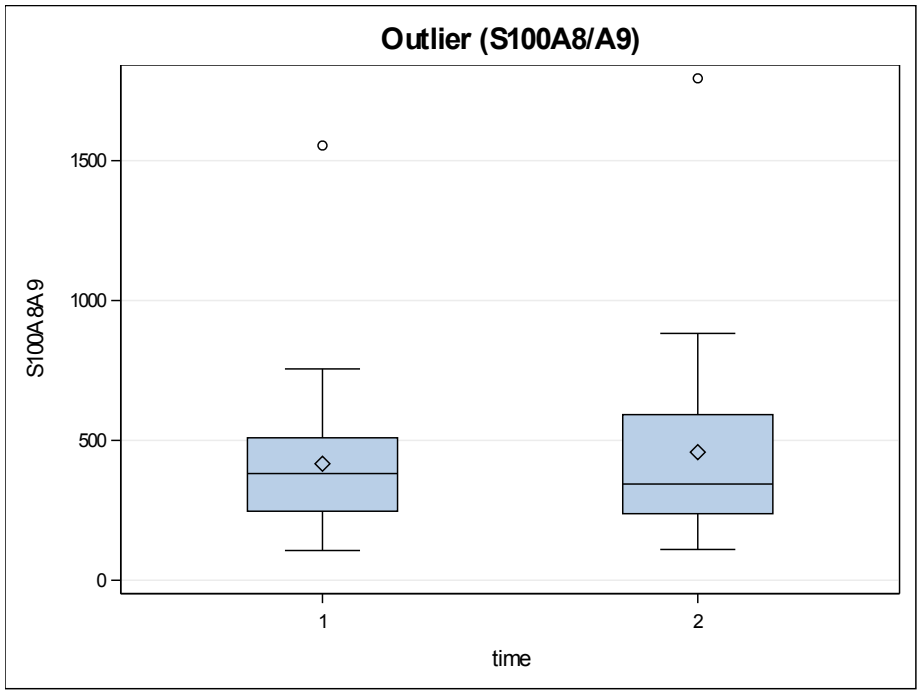
Concentration	LBPP N = 7		Creatine N = 8		Placebo N = 8	
	Baseline	Follow-Up	Baseline	Follow-Up	Baseline	Follow-Up
S100A8/A9	470.49 ± 165.39 (262.6 – 755.5)	487.77 ± 242.72 (199.9 – 882.4)	285.12 ± 138.35 (109.5 – 501.1)	340.56 ± 190.25 (110.1 – 747.9)	387.15 ± 150.03 (190.7 – 541.45)	382.05 ± 213.99 (151.5 – 784.4)
CRP	3.048 ± 1.715 (0.08 – 5.69)	2.104 ± 1.850 (0.35 – 5.37)	1.985 ± 1.578 (0.08 – 5.69)	1.852 ± 1.872 (0.18 – 5.44)	2.316 ± 1.847 (0.24 – 5.46)	1.926 ± 1.429 (0.35 – 4.30)
IL-1β	0.1521 ± 0.0944 (0.04 – 0.30)	0.1321 ± 0.0786 (0.03 – 0.25)	0.1185 ± 0.0614 (0.03 – 0.21)	0.1265 ± 0.0949 (0 – 0.26)	0.1142 ± 0.0716 (0.03 – 0.21)	0.1209 ± 0.0542 (0.07 – 0.20)
IL-6	1.6464 ± 1.4211 (0.51 – 4.71)	1.1409 ± 0.6410 (0.45 – 2.08)	0.8507 ± 0.5923 (0.35 – 2.23)	1.0440 ± 0.6360 (0.43 – 2.17)	1.9840 ± 2.3640 (0.26 – 7.36)	0.9677 ± 0.3552 (0.35 – 1.45)
TNF-α	0.9185 ± 0.5402 (0.25 – 1.77)	0.9330 ± 0.4503 (0.30 – 1.48)	0.8615 ± 0.5990 (0.45 – 2.18)	0.8636 ± 0.5891 (0.37 – 2.17)	0.7564 ± 0.3252 (0.27 – 1.40)	0.6568 ± 0.2051 (0.34 – 1.00)
COMP	239.21 ± 78.34 (143.8 – 339.0)	236.23 ± 57.53 (145.7 – 310.5)	215.14 ± 33.96 (155.0 – 272.0)	238.26 ± 38.97 (196.6 – 294.0)	192.15 ± 64.33 (88.3 – 278.4)	215.56 ± 58.26 (125.7 – 305.4)

Table 6a. Bloodwork – Summary of the Significance in Longitudinal Analysis

Dependent Variables	**Independent Variables**	**Amount of Change**	**P value**
S100A8/A9 (μg/L)	BMI (per 1 kg/m^2 increased)	17.34	0.0144 *
CRP (mg/L)			
IL-1β (ng/L)	Gender (Male – Female)	-0.063	0.0297 *
	BMI (per 1 kg/m^2 increased)	0.005	0.0620
IL-6 (ng/L)			
TNF-α (ng/L)	Affected (Unilateral – Bilateral)	0.338	0.0714
COMP (μg/L)	Time (Follow-up – Baseline, All samples)	24.27	0.0457 *

*$p < 0.05$, **$p < 0.01$

Figure 1. Outlier (S100A8/A9)

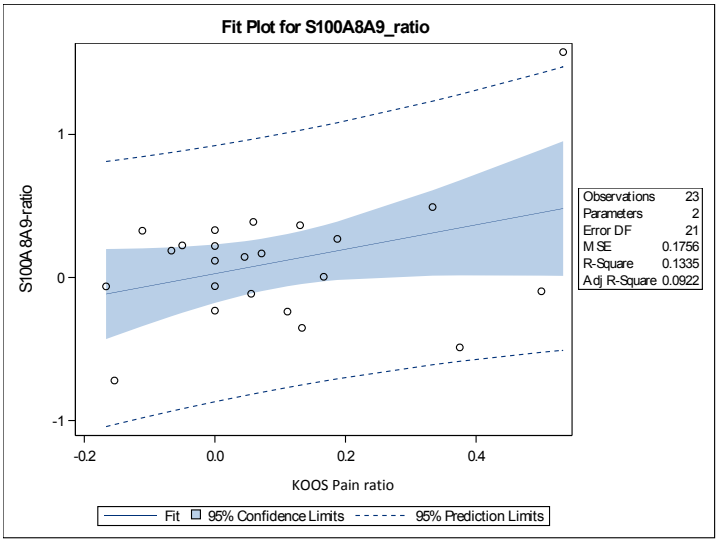


Time 1: Baseline, 377.03±163.10 µg/L (range: 109.53 – 75.50)

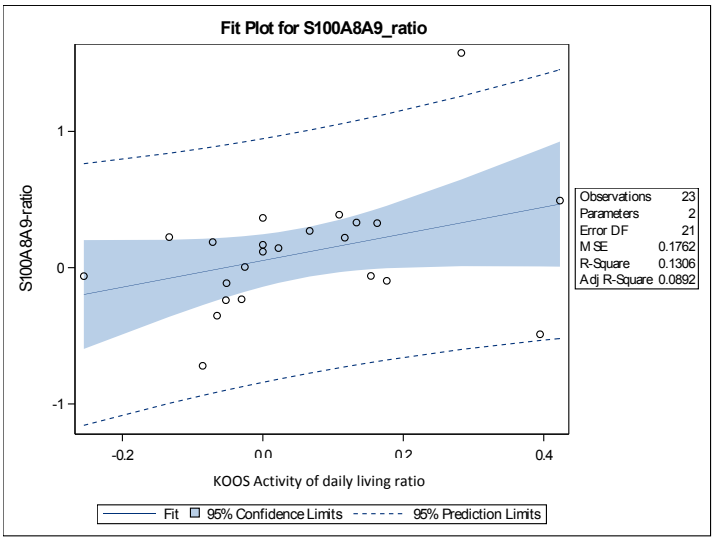
Time 2: Follow-up, 399.80±214.49 µg/L (range: 110.08 – 882.41)

One sample reported outlier both at baseline and follow-up. This case was removed before statistical analysis.

Figure 2. Change ratio (marginal) Significant Correlations – S100A8/A9



S100A8/A9 vs. KOOS-Pain: p = 0.0865, r = 0.365

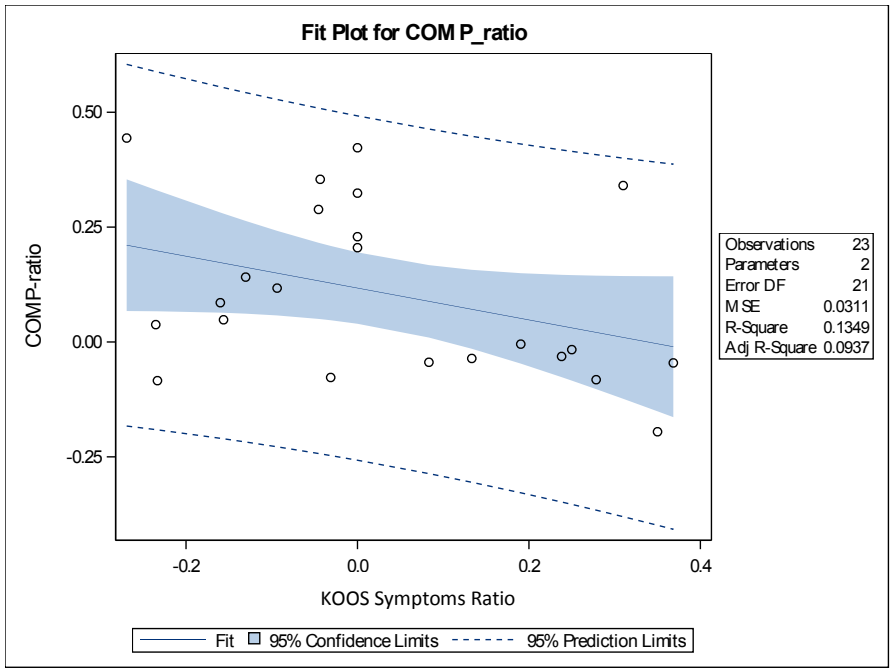


S100A8/A9 vs. KOOS-Activity of daily living: p = 0.0902, r = 0.361

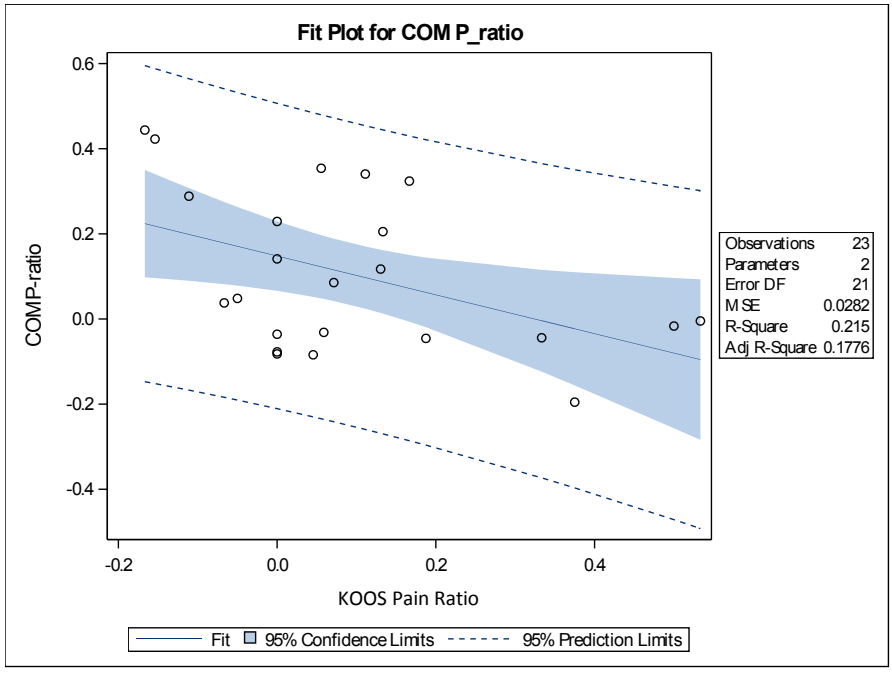
95% Confidence limits: Distribution of fitted line
95% prediction limits: Distribution of population

The S100A8/A9 was proportionally elevated with the increased scores of KOOS-P and KOOS-A at marginal significance.

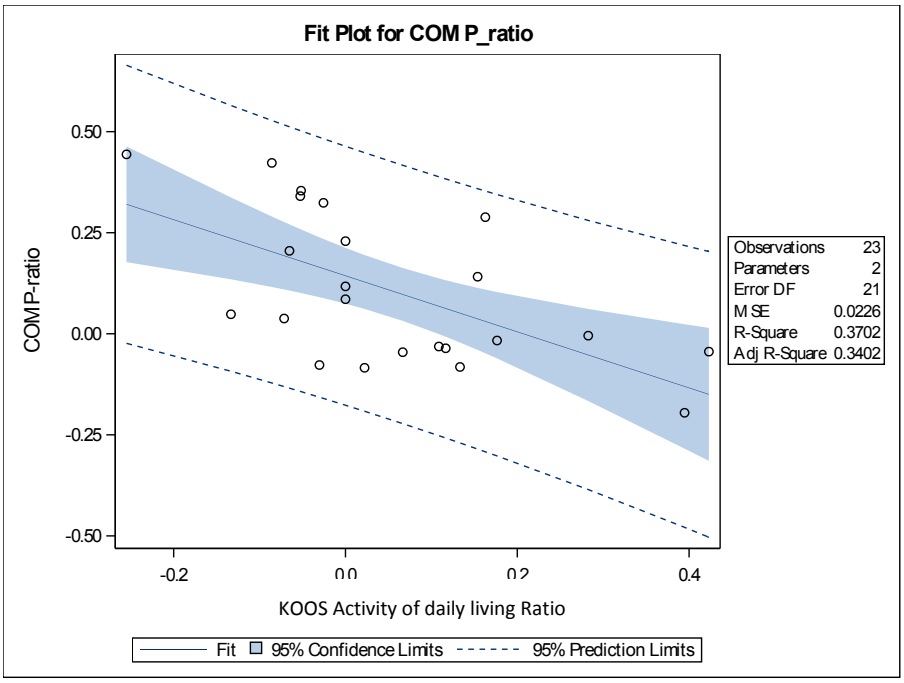
Figure 3. Change ratio (marginal) Significant Correlations – COMP



p = 0.0847, r = -0.367



p = 0.0259, r = -0.464



p = 0.0021, r = -0.608

95% Confidence limits: Distribution of fitted line
95% prediction limits: Distribution of population

The COMP was proportionally down-regulated with the increased scores of KOOS-Symptom (p=0.0847), KOOS-Pain (p=0.0259) and KOOS-Activity of daily living (p=0.0021).

Figure 4. Alter-G Anti-Gravity Treadmill

Figure 4: walking on the Alter-G Anti-Gravity treadmill

Figure 5. Neoprene Shorts for Alter-G Treadmill

Figure 5: wearing a pair of neoprene shorts, to create an airtight seal with the air chamber on treadmill

7. DISCUSSION

The primary objectives of this study included: 1. Determine the concentration of serum S100A8/A9 in individuals diagnosed with mild to moderate knee OA; 2. Determine whether serum S100A8/A9 levels in individuals diagnosed with mild to moderate knee OA were affected by a 12 week low-load walking exercise intervention or dietary supplementation using creatine monohydrate; and 3. Determine whether a relationship exists between KOOS scoring (pain and symptoms) and serum S100A8/A9 levels in individuals diagnosed with mild to moderate knee OA. There were also two additional objectives related to the serum concentration of COMP. We hypothesized that serum S100A8/A9 level was related to the progression of inflammation in knee OA, and that the two interventions would be effective for managing symptoms associated with knee OA. Overall, our findings suggested that creatine supplementation improved knee OA patient's quality of life. LBPP exercise promoted sport and recreation function. Similar to the inflammatory levels for general OA found in previous studies, the concentrations of inflammatory biomarkers remained in normal range. Beyond this, LBPP exercise and creatine supplementation were not able to alter serum levels of inflammatory biomarkers. However, we did observe several correlations between the biomarkers and KOOS scoring.

7.1 Demographic and Anthropometric Data

The target population in this study was senior patients with mild to moderate symptomatic knee OA who were overweight. The average age of participants was 57 years old (ranging from 56.7 to 58.8). Previous studies have identified that aging is a

strong risk factor for knee OA, and older populations generally show higher inflammatory biomarkers and are impacted by joint tissue degradation (Felson et al., 2000; Y. Zhang & Jordan, 2008). Both SF-12 and VAS scales were utilized to show that all participants presented with symptomatic knee OA.

The BMI of the participants was calculated based on body weight and height measured at the Pan Am Clinic. The average BMI was 30.97 kg/m^2 ranging from 29.35 to 32.63 kg/m^2 in each group (>25 kg/m^2). High BMI is also an important risk factor for knee OA (Felson et al., 2000). The other information (i.e. age and gender) provided in the demographic data also reinforced the observations of previous studies regarding a knee OA population (Arden & Nevitt, 2006; Y. Zhang & Jordan, 2008). Each of the groups was homogeneous and representative of an older, overweight patient population, with mild to moderate symptomatic knee OA. As such, the findings of this investigation are generalizable to the defined population.

7.2 Joint Range of Motion

We assessed the joint range of motion in the only the affected knees. The ROM in healthy individuals has been reported as: Hip Flexion 90–135°, Extension 10-30°; Knee Flexion 130–140°, Extension 5–10°; Ankle Plantar flexion 40–50°, Dorsi flexion 15–20° (Medicine, 2013). In our study, the ROMs in patients with mild to moderate knee OA were: Hip Flexion 78 - 92°, Extension 8 – 11°; Knee Flexion 105– 118°, Extension 0.6 – 1.8°; Ankle Plantar flexion 35 – 38°, Dorsi flexion 10 – 16°. All six ROMs were more limited in knee OA participants than a healthy population. Similar results were found in a previous study, where active knee ROMs in obese knee OA patients were reported as

117.9° and 9.7° in flexion and extension, respectively (Masiero et al., 2017). In another investigation, the knee flexion and extension were reported as 122.8° and 2.2° respectively, and the hip extension was 7.8° (demographic missed age and BMI) (T.-J. Wang, Belza, Elaine Thompson, Whitney, & Bennett, 2007). This observation is common in knee OA population, and may be caused by the mechanical changes inside the joint and the weakening of muscles surrounding the joint.

Furthermore, our data suggests that 12-weeks of creatine supplementation was able to decrease the range of motion for the ankle dorsi-flexion ($p = 0.0513$) by 3.77°. However, the result was not deemed to be clinically significant (change in ROM > 5°) (Masiero et al., 2017), and it is possible that this change was simply due to a measurement error. If this decrease truly exists, it is possible that this small change may have occurred as a result of changes (i.e. tightness) in the muscles of the posterior calf that were associated with muscle hypertrophy. Unfortunately, this cannot be confirmed because calf diameter was not measured as part of our study protocal.

There were greater numerical changes observed, but no significance was found in the longitudinal multilevel model. The hip flexion after the 12-week LBPP exercise was decreased from 92.0° to 78.1° (-13.9°); the average knee flexion in the control group increased by 5.12° during follow-up. The relative large standard deviations (15.42 and 22.83 in LBPP hip flexion, and 10.27 and 9.19 in Control knee flexion) rendered the changes insignificant.

Seven independent variables were controlled in the longitudinal analysis, and the influence of these factors on each joint movement was revealed. First, sex affected the range of hip flexion (male vs. female: -13.8°) and knee extension (male vs. female: 0.05°).

BMI appeared to be involved in three movements: hip flexion ($p = 0.0809$, 1.1°), knee flexion ($p = 0.0002$, -0.9°), and ankle plantar-flexion ($p = 0.0520$, 0.6°). Age also affected hip extension ($p = 0.0033$, -0.3°) and knee flexion ($p = 0.0108$, -0.5°). However, most of the influence affected changes were less than 5°, and thus not clinically significant.

Why and how the ROMs were influenced by factors is very complicated, and no previous study investigated their interactions simultaneously. Accumulating research has identified multiple factors associated with ROM: obesity is a negative factor related to the ROM after TKA (Sun & Li, 2017); inflammation was observed with restricted ROM at frozen shoulder (Cher et al., 2017); the hip ROM is found to be different in young male and female (Cheatham, Hanney, & Kolber, 2017); muscle strength played a role involving the movement patterns (Bennell et al., 2008); the property of synovial fluid was correlated with joint flexibility (Ogawa et al., 2017); different types of physical therapies had different effects on joint ROM (Medeiros & Martini, 2018).

To make matters more complicated, one recent article about the ROM after TKA proposed that the young obese group had the same ROM as the old non-obese group (Patel et al., 2017). However, all these factors were overlapped with each other: obesity has great impact on inflammation and mechanical stress and is related to aging; inflammation can damage the joint structure directly, weaken the muscle, change the synovial fluid, and lead to pain at the joint; physical therapies also have uncertain effect on inflammation and the anatomy structure.

7.3 Pain VAS for Full Weight Bearing Walking

We used a Visual Analog Scale to trace how much pain the participants reported during the 30-minute full weight bearing walking. The experiment was designed to show the level of pain in daily walking. The clinical significant difference for this scale was reported from 0.9 mm to 1.1 mm for mild pain (Kelly, 1998).

The improvement of VAS in the LBPP exercise group and creatine supplementation group were 1.37 and 0.92 respectively after 12-week intervention, however neither of these two improvements was confirmed by longitudinal analysis. By contrast, sex, number of affected knees, K&L grade, and BMI may affect the pain VAS score at marginal significance ($p = 0.0780 – 0.0985$). The results suggested that knee pain during walking was more severe in females, in unilaterally affected patients, with milder knee OA, and an increased BMI.

The results from a previous study published in 2015 from our laboratory, which recruited 31 participants, were comparable to the LBPP exercise VAS results in this investigation (Peeler et al., 2015). The population in the study was the same: overweight (32.8 vs. 30.97 kg/m^2), older (64 vs. 57 years), and had mild to moderate knee OA. After the same walking strategy on LBPP treadmill for 12 weeks, the VAS score in the previous study was significantly reduced from 2.6 to 1.8 at $p = 0.01$ level with paired-t test (Peeler et al., 2015). The observed discrepancy between the two studies may be due to: 1. small sample size leads to a higher change of false negative; and 2. the different statistical analysis methods: factors such as number of affected knees, BMI, and gender were not able to be statistically controlled in repeated ANOVA in previous study, and thus increased the chance of confounding bias.

Therefore, expanding the sample size and controlling for more factors in further research may increase chance to support the effects of LBPP exercise and creatine supplementation on relieving pain during walking.

7.4 KOOS Score

Currently, KOOS is the questionnaire most commonly used by physicians to track knee OA progression and subsequently adjust treatment options. The five subscales contain 42 questions to assess symptoms, pain, activity of daily living, sport and recreation function, and quality of life. Each question is scored from 0 to 4 (E. M. Roos et al., 1998). Similar to the VAS, KOOS also accounts for the psychometric response. The minimal detectable changes in subscores are different depending on the disorder, such as TKA, knee OA, and ACL injury. It is also different in clinical use compared to research. The minimal detectable change for knee OA study have been set to: Pain 13.4, Symptoms 15.5, ADL 15.4, Sport/Rec 19.6, and QOL 21.1 (Natalie J. Collins et al., 2011). The reliability of KOOS on evaluating the disability in knee OA has been well established.

From our interventions, one statistical significance ($p = 0.0467$) was observed in the score of QOL in Creatine group, where the 12-week creatine supplementation improved the KOOS-QOL score by 12.5. However, the minimal detectable difference for this category is 20 for older population (N. J. Collins et al., 2016). One marginal statistical difference ($p = 0.0533$) was seen in the Sport/Rec score, where the LBPP group had an increase of 17.7, close to the minimal detectable change of Sport/Rec (19.6). Sex also had a role in Sport/Rec scores with males reporting 20.1 points higher than females ($p = 0.0231$).

Interestingly, the severity of knee OA (K&L grade) was a strong indicator of the KOOS scores. The functions were generally worse in the milder knee OA participants with Grade II: -9.8 points was found in Symptoms ($p = 0.0670$), -16.2 points in ADL ($p = 0.0071$), -21.5 points in Sport/Rec ($p = 0.0146$), and -13.3 points in QOL ($p = 0.0473$). This was consistent with our VAS pain results. The reason why milder knee OA patients suffer more remains unknown. One possible explanation could be psychological factors and the symptom tolerance: in the early stage of OA (K&L grade II) or unilateral affected knee OA, the patients are more sensitive to the signs and symptoms, and thus they intend to reduce the symptoms by limiting their activity and quality of life.

Different from VAS evaluation which showed the immediate pain during walking, the result of KOOS illustrates the overall disability of knee OA participants in the past 12 weeks in all activities (E. M. Roos et al., 1998). The KOOS pain score was not correlated to any of our test indicators. Contrary to results found in creatine supplementation in a previous study (Neves et al., 2011), the increased muscle strength did not help improve pain in our participants.

In a previous study, all of the five KOOS subscales showed statistical significance after the same 12-week LBPP exercise, which was analyzed by paired t-test (Peeler et al., 2015). The Sport/Rec also showed the greatest changes (22) among the five subscales, which is the same trend in our study; and another great numerical change was seen in QOL (12), which was not illustrated in present study. As with the VAS, these differences could be caused by our study's small sample size and different statistical analysis.

The suggested minimal detectable changes may not be applicable to our study. The minimal changes reported in previous studies were calculated on numerous

published articles though meta-analysis (N. J. Collins et al., 2016; Natalie J. Collins et al., 2011). However, the authors admitted that the variables considered in meta-analysis were not detailed enough to distinguish the different aims of studies, for example the surgical and non-surgical intervention were mixed; which means the minimal detectable changes were just for reference in academic investigations (N. J. Collins et al., 2016). In other words, the inclusion criteria and variables are much more designated in specific studies, and then the minimal detectable changes could be minor in studies to show clinical significance. In future research, we need more details from the previous meta-analysis and more advanced statistical tools to calculate more suitable minimal detectable changes based on the degree of freedom.

7.5 Concentration of Biomarkers

Serum S100A8/A9 concentration was elevated with increasing BMI ($p = 0.0144$): per 1 kg/m^2 increase in BMI, serum S100A8/A9 elevated 17.34 μg/L. Previously, studies have also shown that S100A8/A9 increased with higher BMI (Catalán et al., 2011) (Mortensen et al., 2009).

Serum COMP level were also increased by 24.27 μg/L on average after 12 weeks ($p = 0.0457$), and neither LBPP nor creatine supplementation changed the trend. In a previous study, it has been discovered that elevated COMP is a risk factor for hip OA (Chaganti et al., 2008).

The biomarkers tested in this study include S100A8/A9, pro-inflammatory (IL-1β, TNF-α), anti-inflammatory (IL-6), dying cells marker (CRP), and mechanical stress marker (COMP). Surprisingly, no significant changes were observed over the

interventions. As compared to the normal range of these biomarkers, most biomarker levels did not differ from those found in a healthy population. CRP was the only biomarker with a slightly elevation compared to a healthy population (Pepys & Hirschfield, 2003; Tracy et al., 1997).

	S100A8/ A9 (μg/L)	CRP (mg/L)	IL-1β (ng/L)	IL-6 (ng/L)	TNF-α (ng/L)	COMP (μg/L)
Normal Serum Concentration	30 – 910	1.73 – 2.32	0 – 3.9	0.447 – 9.96	0.550 – 0.816	66.8 - 490

Furthermore, a US study (NCT00305890) published in 2016 conducted analysis on participants with an average age of 58.5 years and BMI of 33.9 kg/m^2. Fifty-four percent (54%) of the participants in control group had mild to moderate knee OA (Huebner et al., 2016). The means of each biomarker in this group were reported as: high sensitive CRP 6.727 mg/L; IL-1β 397 ng/L; and IL-6 1.69 ng/L. The TNF-α was excluded due to "only 31% yielded measurable concentrations" (Huebner et al., 2016).

Another investigation from the Netherlands investigated the relationship between S100A8/A9 levels and clinical characteristics of patients including pain, stiffness, function, CRP, ESR, and osteophytes (Mahler et al., 2015). Based on the 57 knee OA patients examined, the average serum level S100A8/A9 was reported as 335 μg/L. Eighty-nine percent (89%) of the participants had < 5 mg/L serum CRP concentration (Mahler et al., 2015). Although the published results were for both hand and hip OA, direct correspondence with the author indicated that results specific to knee OA did not show a difference. CRP is the only biomarker that showed a difference in these studies, and this may have occurred because of a different average BMI of the participants (33.9

vs. 27.5 vs. 30.97 kg/m^2), as obesity has been linked to elevated levels of CRP (Firdous, 2014).

Regarding the biomolecular function of S100A8/A9 with low serum concentration (< 5 mg/L), it seems that the S100A8/A9 in a knee OA population could be more related to a protective function, which can promote cell proliferation in tissue/cell culture (Ghavami et al., 2009). However, the serum is different from the synovial fluid and interstitial fluid: serum concentration evaluates a systemic inflammation; synovial fluid evaluates a generally local inflammation; but each chondrocyte sits in cartilage lacuna and its inflammation level should be evaluated by interstitial fluid. Therefore, the serum concentration cannot accurately present the dose of S100A8/A9 around chondrocytes, and it is still possible that S100A8/A9 promotes apoptosis on chondrocyte, which could be a trigger of osteoarthritis. With this in mind, future investigations could use histological quantitative examination in an animal model or from TKA specimens with knee OA to clarify this question.

In summary, the change in serum S100A8/A9 correlated to the change in BMI, and the COMP level was elevated over time in our study. The levels of other biomarkers, including IL-1β, and IL-6, were within normal ranges and did not change significantly in our population over the course of the investigation (Garcia-Arias et al., 2013; Weissmann & Korchak, 1984).

As such, our experimental results do not support the use of serum level of S100A8/A9 as an effective biomarker for tracking inflammatory progression associated with knee OA.

In theory, S100A8/A9 could be an effective biomarker for tracking knee OA progression, as it has been shown to play a role in inflammation. Specifically, S100A8/A9 concentration is related to both chondrocytes metabolism and inflammatory response (Zreiqat et al., 2007, p. 9). But, based on the findings of this investigation, the pathways of S100A8/A9 as they relate to inflammation may be more complicated than first thought.

There are several reasons that may account for negative results observed. First of all, the amount of released S100A8/A9 may not have been high enough to influence its concentration in the serum. The predicted concentration of S100A8/A9 required to induce apoptosis should be higher than 100 μg/ml. However, 50-100 μg/ml of S100A8/A9 can be completely neutralized by 10 μg of Zinc sulfate or $Cu^{2+,}$ $Mn^{2+,}$ and Fe^{2+} (Yui et al., 2003). Evidence suggests that Zinc combines with extracellular S100A8/A9, which inhibits its apoptosis function, but does not competitively block the receptor of S100A8/A9.

Currently, no previous studies have identified the amount of S100A8/A9 per cell or per unit of tissue. From a previous study on arthritis published in 2012, researchers found on average 450 cells/mm^3 white blood cells in OA patients' synovial fluid; out of those, only 2% (9 cells/mm^3) were neutrophils. In RA patients, 11000 cells/mm^3 WBC were found, and about 81.5% (8965 cells/mm^3) were polymorphonuclear leukocytes. In psoriatic arthritis patients, the number of polymorphonuclear leukocytes in synovial fluid was about 2653 cells/mm^3 (Oliviero et al., 2012). These studies provide clues about the aggregation of polymorphonuclear leukocytes in synovial fluid in inflammatory arthritis (RA and psoriatic arthritis), and this may be the reason for the elevation in inflammatory

biomarkers, including S100A8/A9. Furthermore, the serum CRP level in OA participants was reported at 3.77 mg/L. These results may imply that the role of inflammation in OA is distinct from inflammatory arthritis.

Beyond this, the universality of S100A8/A9 physiological distribution was ignored. S100A8/A9 has been suggested as a diagnostic method for RA, coronary artery disease, systemic lupus erythematosus, and Crohn's disease (Guo et al., 2015; Kopeć-Mędrek et al., 2016; Meuwis et al., 2013). In the case of OA, previous animal studies suggest that the S100A8/A9 might be valuable for early diagnosis of OA (R. F. P. Schelbergen et al., 2016; Zreiqat et al., 2010). However, the serum concentration of S100A8/A9 can be easily influenced by the fluctuation of innate immune system through events such as infection, cardiovascular disorder, or arthritis at other joints (Guo et al., 2015; Stacey, Gibala, Martin, & Timmons, 2010), which were not controlled. It is hard to conclude that such a widely produced protein in myeloblasts can be used as a biomarker for a specific disease, unless there is an excess of serum S100A8/A9 combined with other clinical indications.

Finally, the physiological changes in knee OA may not be apparent within a 12-week intervention. In the previous study, we have observed the changes in KOOS and VAS pain after 12-weeks of LBPP walking (Peeler et al., 2015). To our knowledge, the serum biomarkers in OA did not change significantly within the 12 weeks, and mechanical stress placed on the knee joint during exercise was not quantified. To date, only 1 other study has observed the progress of OA for a longer period in an animal model (Zreiqat et al., 2010). This investigation reported that on the load-bearing regions of the affected joint, there were reduced staining of S100A8/A9 of chondrocytes at the

later stage of OA; but at the joint marginal region, chondrocytes showed the same level of S100A8 at all stages in all the groups. These results suggest that the expression of S100A8 and S100A9 may be regulated more by mechanical stress. Finally, the authors stated that, S100A8 and S100A9 may play a role or be produced in the initial degeneration or early stage of OA; and in the case of OA progression, the two proteins may no longer be effective molecular markers. Further research on the metabolism of S100A8/A9 and their levels relating to exercise stress and aging may provide more clues about their roles in diseases.

7.6 Change ratio Correlations

The relationship between the change ratio of S100A8/A9 and the change in KOOS scores were examined. Two marginally significant low positive relationships were found with KOOS pain ($r = 0.365$, $p = 0.0865$) and KOOS activity of daily living ($r = 0.361$, $p = 0.0902$). It seems that the pain and function of knee OA participants were improved with elevation of serum S100A8/A9.

A previous study illustrated that S100A8/A9 levels in an older overweight knee OA population were not influenced by a patients K&L grade (Mahler et al., 2015). Beyond this, the relationships between serum S100A8/A9 and other outcomes is best illustrated by an odds ratio (OR) or β-coefficient. The value of the OR determines if the factor is protective ($OR < 1$) or harmful ($OR > 1$) (Szumilas, 2010), while the β-coefficient, as well as standardized regression coefficient, is used to evaluate the strength of risk factor (Newman & Browner, 1991).

In a previous study, the relationship between an increase in 1 mg/L serum S100A8/A9 concentration and either the WOMAC (Western Ontario and McMaster Universities Osteoarthritis Index) scores or osteophytes / joint space were examined. Knee pain, stiffness, and function on WOMAC scoring was not associated with serum level of S100A8/A9 ($p = 0.52 – 0.94$); and the positive β-coefficients were also not significant (Mahler et al., 2015). As the KOOS questionnaire is an extension of WOMAC, the WOMAC scores can be compared with KOOS scores (Natalie J. Collins et al., 2011).

K&L grading includes consideration of osteophytes and joint space width (Kellgren & Lawrence, 1957) and previous investigations have reported a negative correlation between osteophytes and the level of S100A8/A9 ($p = 0.06$) (Mahler et al., 2015). This may suggest that the level of serum S100A8/A9 decreases with the progression of knee OA. However, we did not observe a similar negative relationship between K&L and S100A8/A9 in the present study. To extending the period of observation may help reveal this trend.

As an additional objective, COMP as an extracellular matrix protein related to mechanical stress was examined. Its biological function is still not clear, but in some cases the level of COMP may indicate the progression of OA (Bartels et al., 2014). One study in 2005 by Wislowska M reported that the serum concentration of COMP did not reveal a difference between RA and OA (Wisłowska & Jabłońska, 2005). This result suggests that COMP is not an inflammatory biomarker, but may be related to the mechanical stress or damage to articular cartilage.

In our study, we examined the change ratio of COMP with the change ratios of KOOS scores. The results suggest that Pain, Symptoms, and ADL subscores had a

significant negative relationship with COMP levels. The strength of the relationship between COMP and KOOS ADL was moderate ($r = -0.608$, $p = 0.0021$). These findings are consistent with one cohort study published in 2014 which illustrated similar results in serum COMP (Bartels et al., 2014). The demographics of this study's participant population was: 62.6 years in age, 37.1 kg/m^2 in BMI, and average grade 2.6 in K&L (range: 1 – 4). The unit used in serum COMP was U/L. This studies data suggested that a negative correlation existed between changes in COMP and "KOOS-4" ($r = -0.13$, $p = 0.091$) (Bartels et al., 2014). Although the so-called "KOOS-4" was calculated as the average of Pain, Symptoms, Function, and QOL, it still implied a possible relationship between serum COMP and KOOS. As such, it would appear that the change ratio in serum COMP is related to the improvement in disability.

While on one hand, an increase in COMP could be a risk for OA, and the increasing COMP level could be related to knee OA progression. But, on the other hand, the milder knee OA patients with Grade II had a higher COMP concentration (22.58 μg/L) than the moderate knee OA patients with Grade III. No previous studies illustrated this problem, and there was no mechanism that can be adequately used to explain this. What if there was a shifting point in COMP level between K&L Grade II and III: before this point, the COMP may predict OA progression, but after this point, the COMP concentration dropped as OA worsens. Further studies may help to reveal more information about the paradoxical relationship between COMP and OA progression.

7.7 Limitations

There are several limitations to this study, which include participant recruitment, the use of medications, and statistical methodology.

The risk factors of OA were not controlled completely. Participants with and without history of long term trauma were combined in the study and not discussed separately. Also, some criteria of inclusion in this study were based on self-reports, and three participants' BMI was found to be under 25 kg/m^2, which represented a normal body weight. The exercise capacity in each group may be different and was not assessed. And discussion between groups could be impacted by potential compounding biases.

Participants were not asked to stop their NSAIDs for pain relief during the 12-week intervention due to ethical considerations. Twenty (20) of twenty-six (26) participants stayed on NSAIDs and did not change the dose during this study. Anti-inflammatory drugs might have affected the results, especially serum biomarkers. The COX-2 inhibitor is much more efficient and has fewer adverse effects than non-selective COX inhibitor. They are able to competitively inhibit the COX enzyme and reduce the generation of active prostaglandins (PGs), which in turn reduces the level of inflammation. However, the competitive inhibition on COX from NSAIDs can be antagonized by other substances, such as NF-κB. NF-κB is one of the most common inflammatory biomarkers. Therefore, there is a chance that the NSAIDs do not have a great impact on serum S100A8/A9 or other inflammatory biomarkers.

Radiological examination was not performed at follow-up due to cost and safety. As a result, the follow-up outcomes were compared between serum biomarkers and subjective evaluations.

The sample size had a significant impact on the power of our study. We only had 7 or 8 valid individuals for each group, which was not enough to conclude significance in a multilevel longitudinal model. With the increasing complexity of the model, the power decreases with the same sample size. A multi-centre clinical trial that can obtain information from hundreds of patients would help to reveal more significance.

Finally, the statistical analysis could have been further improved. In this study, we created a 2-level longitudinal model with seven independent variables. Based on the medicine knowledge from professors and the statistical designing from statisticians, more accurate model could be established with their cooperation. The most ideal model will include all of the known risk factors and their interactions in the formula, which probably needs millions of cases enrolled into the database.

7.8 Significance and Conclusion

The results of this investigation indicated that LBPP exercise and creatine supplementation may improve the sport/recreation function and quality of life in mild to moderate knee OA population. The key findings of this study are as follows:

- The serum S100A8/A9 concentration in older overweight mild-moderate knee OA population was in normal range.
- The serum level of S100A8/A9 cannot be used to track the progressive inflammation in knee OA patient population.
- The increase of serum S100A8/A9 might be correlated to less pain and better activity of daily living function reported though KOOS questionnaire.

- The decrease of COMP was correlated to less pain, fewer symptoms, and better ADL reported though KOOS questionnaire.
- 12-week LBPP exercise might improve the sport/recreation function in knee OA population.
- 12-week Creatine supplementation was able to improve the quality of life of knee OA patients.

In terms of serum S100A8/A9, this investigation provided the initial analyses combining clinical observation and basic molecular mechanisms. There are many studies that focus on the pathways of S100A8/A9 during early stages of OA in animal models through operation or injection, and others that revealed no role of S100A8/A9 in knee OA patients. However, no study has explored the concentration of S100A8/A9 in vivo and looked into potential biochemical changes in OA patients. We not only quantified the serum S100A8/A9 level in our knee OA population, but also addressed questions about its role in inflammation in knee OA.

Creatine supplementation and LBPP exercise have the potential to be a part of knee OA treatments, with benefits such as low-cost, non-invasiveness and better compliance. The preliminary studies has proved that LBPP exercise had the ability to modify the function in knee OA patients (Peeler et al., 2015; Takacs et al., 2013); creatine supplementation could supress the inflammatory level and improve muscle strength for a long period (Twycross-Lewis et al., 2016). Further studies should focus not only on relieving the pain of patients, but also slowing down the progression of the disease.

Overall, our findings may allow researchers to reconsider the role of S100A8/A9 and COMP in knee OA patients, and further understand the influence from creatine supplementation and LBPP exercise. These results may also provide physicians with better protocol on creatine supplementation and LBPP exercise.

7.9 Future direction

The main question about biomarkers we addressed in this study was their role in inflammation associated with knee OA. The results suggest that inflammation could be localized inside synovial capsule or be inactivated. As such, future investigations should focus on the biomarkers in synovial fluid.

For the clinical application, the effectiveness of LBPP exercise and creatine supplementation need to be further investigated to identify the benefits and risks of these two cost-efficient interventions. More rigorous randomized clinical trial with larger sample sizes should be employed.

In summary, our results confirm that knee OA is a multi-factorial disease. Just like a dam, a break can occur at any weak point, and spread to all other parts. It is not possible to find out the source with a narrow viewpoint. Further research is necessary to reveal the mechanism of OA in a broader scope.


Mechanical
Age
Obesity
Inflammation
Gender
Synovial
Muscle strength

8. APPENDIX

8.1 Participant Consent Form

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: *Quantifying Inflammation Associated with Mild – Moderate Knee Osteoarthritis: Serum S100A8/A9*

Protocol number: "_____"

Principal Investigator: *Jason Peeler, 130 Basic Medical Science Building, 745 Bannatyne Avenue, Winnipeg, MB, (204) 272-3146*

Co-Investigator: *Stephen Cornish, 117 Frank Kennedy Centre Winnipeg, MB, 204-474-9981*

Sponsor: *Pan Am Clinic Research Centre, 75 Poseidon Bay Winnipeg, MB*

You are being asked to participate in a Clinical Trial (a human research study). Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

The study doctor is receiving professional fees and financial support to conduct this study.

Purpose of Study

This Clinical Trial is being conducted to study *the novel diagnosis method and treatments of knee osteoarthritis.* **You are being asked to take part in this study because you have** *met our inclusion criteria: 1. Ages 45-65; 2. BMI over 30 kg/m^2; 3. Mild to moderate knee osteoarthritis; 4. Knee pain during normal daily activities; 5. Without history of diseases or trauma which is considered have influence in this study.* **A total of** *54* **participants will participate in this study.**

The purpose of this study is to *further our understanding of how inflammatory biomarkers can be used to accurately diagnose knee OA, trace disease progression, assist in the development of screening protocols for a high-risk groups, and determine the efficacy of treatment commonly used in the battle against knee OA in North Americas aging population***.**

This research is being done because *the current diagnosis cannot accurately reflect the pathogenesis progression of knee OA, and lead to the lack of effective treatments***.**

Study procedures

In this study, you will be "randomized" into one of *three* **study groups described below. "Randomized" means that you are put into a group by chance, like flipping a coin. You will have a** *one in three* **chance of being placed in any group** *by simple randomization method. Three groups will be set in this study: Exercise group, Creatine supplementation group, and Control supplementation group.*

Neither you nor the study doctor will know which arm of the study you will be in. As the strategy of Exercise group is obviously different from the supplementation groups, the participants in Exercise group are not blinded. The other participants, who are in Creatine or Control supplementation groups, will be single-blinded; only doctor will know which group you are in. In an emergency, this information will be made available.

If you take part in this study, you will have the following tests and procedures:

Questionnaire: You will have to answer the SF-12 and IKDC questionnaires as prescreening to learn your general health status; KOOS questionnaire to quantify the status of knee joint for the baseline and outcome evaluation.

Body anthropometry will be applied as part data of baseline.

The blood sample will be taken twice, approximate 20 mL each time before and after the 12-week intervention respectively for baseline and outcome evaluation.

The 12-week interventions for each group list as below:

1. Exercise group: Participants will walk at 3.1 mph/ 0 incline for 30 minutes, 3 times per week on an Alter-G treadmill in Pan Am Clinic. Participants will be blinded to the parameters of the treadmill. A written record will be saved for each walking.

2. Creatine supplementation group: Participants will supplement their regular diet with creatine monohydrate as 5 grams, 4 times per day in the first week; and then, 5 grams per day as a maintenance dose for the remaining 11 weeks.

3. Control supplementation group: Participants will supplement their regular diet with an inactive substance (maltodextrin) as 5 grams, 4 times per day in the first week; and then, 5 grams per day as a maintenance dose for the remaining 11 weeks.

Participation in the study will be for *13-weeks' follow-up***.**

The researcher may decide to take you off this study if *you meet some personal circumstances or this research is not suitable for continuing, including but not limited to: 1. Participant's medical best interest; 2. Serious side effects happen; 3. Failure to follow the interventions as described; 4. Funding is stopped; 5. Drug supply is insufficient; 6. Participant's condition worsens; 7. New information becomes available, and etc.*

You can stop participating at any time. However, if you decide to stop participating in the study, we encourage you to talk to the study staff and your regular doctor first.

The individual and aggregate results will be provided to the participants as feedback in the end of this study, after statistical analysis. You can choose the method receiving these data.

Risks and Discomforts

While on the study, you are at risk for certain side effects:

1. Increased knee pain or soreness; 2. Muscle strain during exercise intervention; 3. Slight pain or bruising with blood collection procedure; 4. Chance of infection from blood draws, and etc.

There are possible side effects related to the intake of creatine:

1. nausea and/or stomach pain; 2.diarrhea; 3. Weight gain; 4. Muscle cramps; 5. Dehydration symptoms and electrolyte imbalance; 6. Abnormal heart rate, and etc.

There also may be other side effects or discomforts that we cannot predict. There is lack of sufficient data on the use of creatine during pregnancy. Although in theory, the creatine supplementation may help the nursing mother to avoid creatine deficiency syndromes, it is still suggested to be avoided.

Your condition may not improve or may worsen while participating in this study. If you are in the supplementation group that receives inactive substance, your symptoms or condition may worsen or not improve.

All supplementation products must be kept out of the reach of children and persons of limited capacity to read or understand.

Benefits

By participating in this study, you will be providing information to the study doctors that will show the effects of *moderate exercise or creatine supplementation* **for the treatment of** *knee osteoarthritis***. There may or may not be direct medical benefit to you from participating in this study. We hope the information learned from this study will benefit other participants with** *knee osteoarthritis* **in the future.**

Costs

All clinic and professional fees, diagnostic and laboratory tests which will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Payment for participation

You will receive no payment or reimbursement for any expenses related to taking part in this study.

Alternatives

Instead of being in this study, you may request the standard medical treatment for *knee Osteoarthritis.*

You do not have to participate in this study to receive treatment for your condition. Please talk to your regular doctor about all your treatment options.

Confidentiality

Information gathered in this research study may be published or presented in public forums, however your name and other identifying information will not be used or revealed. All study documents related to you will bear only your assigned code and initials. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law.

The University of Manitoba Biomedical Research Ethics Board may review research-related records for quality assurance purposes.

All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your medical/research records need to be copied to any of the above, your name

and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave the *Pan Am Clinic Research Centre.*

With your permission your Family Physician (GP) will be notified about your participation in this study.

Voluntary Participation/Withdrawal from the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision not to participate or to withdraw from the study will not affect your other medical care at this site. If your study doctor feels that it is in your best interest to withdraw you from the study, your study doctor will remove you without your consent.

We will tell you about any new information that may affect your health, welfare, or willingness to stay in this study.

Medical Care for Injury Related to the Study

In the case of injury or illness resulting from this study, necessary medical treatment will be available at no additional cost to you.

You are not waiving any of your legal rights by signing this consent form nor releasing the investigator(s) or the sponsor(s) from their legal and professional responsibilities.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research participant. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff: *Dr. Jason Peeler* **at** *(204) 272-3146.*

For questions about your rights as a research participant, you may contact The University of Manitoba Biomedical Research Ethics Board at (204) 789-3389.

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent

I have read this consent form. I have had the opportunity to discuss this research study with *Jason Peeler* and or his/her study staff. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I believe that I have not been unduly influenced by any study team member to participate in the research study by any statement or implied statements. Any relationship (such as employee, student or family member) I may have with the study team has not affected my decision to participate. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of my medical records by *Pan Am Clinic Research Centre*, the Food and Drug Administration, the Health Protection Branch, government agencies in other countries, and The University of Manitoba Biomedical Research Ethics Board.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

I agree to being contacted in relation to this study.
Yes □ No □

I agree to my family physician being notified of my participation in this study.
Yes □ No □

Participant signature_____ **Date** _____

(day/month/year)

Participant printed name: _____

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent

Printed Name: _____ **Date** _____

(day/month/year)

Signature: _____

Role in the study: _____

8.2 Knee Injury and Osteoarthritis Outcome Score (KOOS)

KOOS KNEE SURVEY

Today's date: _____/_____/_____ Date of birth: _____/_____/_____

Name: _____

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.

Symptoms
These questions should be answered thinking of your knee symptoms during the **last week**.

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
☐	☐	☐	☐	☐

Stiffness
The following questions concern the amount of joint stiffness you have experienced during the **last week** in your knee. Stiffness is a sensation of restriction or slowness in the ease with which you move your knee joint.

S6. How severe is your knee joint stiffness after first wakening in the morning?

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

S7. How severe is your knee stiffness after sitting, lying or resting **later in the day**?

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

Pain

P1. How often do you experience knee pain?

Never	Monthly	Weekly	Daily	Always
☐	☐	☐	☐	☐

What amount of knee pain have you experienced the **last week** during the following activities?

P2. Twisting/pivoting on your knee

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P3. Straightening knee fully

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P4. Bending knee fully

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P5. Walking on flat surface

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P6. Going up or down stairs

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P7. At night while in bed

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P8. Sitting or lying

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

P9. Standing upright

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

Function, daily living

The following questions concern your physical function. By this we mean your ability to move around and to look after yourself. For each of the following activities please indicate the degree of difficulty you have experienced in the **last week** due to your knee.

A1. Descending stairs

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A2. Ascending stairs

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

For each of the following activities please indicate the degree of difficulty you have experienced in the **last week** due to your knee.

A3. Rising from sitting

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A4. Standing

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A5. Bending to floor/pick up an object

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A6. Walking on flat surface

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A7. Getting in/out of car

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A8. Going shopping

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A9. Putting on socks/stockings

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A10. Rising from bed

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A11. Taking off socks/stockings

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A12. Lying in bed (turning over, maintaining knee position)

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A13. Getting in/out of bath

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A14. Sitting

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A15. Getting on/off toilet

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

For each of the following activities please indicate the degree of difficulty you have experienced in the **last week** due to your knee.

A16. Heavy domestic duties (moving heavy boxes, scrubbing floors, etc)

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

A17. Light domestic duties (cooking, dusting, etc)

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

Function, sports and recreational activities

The following questions concern your physical function when being active on a higher level. The questions should be answered thinking of what degree of difficulty you have experienced during the **last week** due to your knee.

SP1. Squatting

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

SP2. Running

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

SP3. Jumping

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

SP4. Twisting/pivoting on your injured knee

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

SP5. Kneeling

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

Quality of Life

Q1. How often are you aware of your knee problem?

Never	Monthly	Weekly	Daily	Constantly
☐	☐	☐	☐	☐

Q2. Have you modified your life style to avoid potentially damaging activities to your knee?

Not at all	Mildly	Moderately	Severely	Totally
☐	☐	☐	☐	☐

Q3. How much are you troubled with lack of confidence in your knee?

Not at all	Mildly	Moderately	Severely	Extremely
☐	☐	☐	☐	☐

Q4. In general, how much difficulty do you have with your knee?

None	Mild	Moderate	Severe	Extreme
☐	☐	☐	☐	☐

Thank you very much for completing all the questions in this questionnaire.

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