THE EFFECT OF FREEZE DRIED WHOLE BLUEBERRIES ON PAIN, GAIT PERFORMANCE AND INFLAMMATION IN INDIVIDUALS WITH SYMPTOMATIC KNEE OSTEOARTHRITIS

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ABSTRACT

THE EFFECT OF FREEZE DRIED WHOLE BLUEBERRIES ON PAIN, GAIT PERFORMANCE AND INFLAMMATION IN INDIVIDUALS WITH SYMPTOMATIC KNEE OSTEOARTHRITIS

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Objective: The purpose of this study was to examine the effect of freeze dried whole blueberries on pain, gait performance, and inflammation in individuals with symptomatic knee osteoarthritis (OA).

Methods: A total of 63 men and women with symptomatic knee OA were recruited and randomized into either a treatment group (blueberry group) or placebo group, for a period of 4 months (120 days). The treatment group (n = 33) consumed 40 grams of freeze dried whole blueberry powder divided into two parts per day (each 20-gram packet was reconstituted with 6-10 oz water). The placebo group (n=30) consumed 40 grams of control powder also divided into two parts per day, which was composed of a mixture of maltodextrins and fibers to mimic the carbohydrate composition of whole blueberries, but without whole blueberry content. The appearance of the placebo powder, along with energy content, are similar to the blueberry powder. Demographic information including weight, height, and blood pressure (systolic and diastolic) were collected at baseline, midpoint (60 days), and final point (120 days) visits. Western Ontario

McMaster Osteoarthritis Index (WOMAC) questionnaires were conducted at the baseline, midpoint (60 days), and final point visit (120 days). Gait analysis was also performed at these time points. Additionally, overnight fasting venous blood samples were collected at baseline, midpoint, and final visits to assess biomarkers of inflammation. Treatment effects were examined with repeated measures analysis of variance (ANOVA).

Results: A total of 49 participants completed the study with an attrition rate of 22%. WOMAC total score and sub-groups, including pain, stiffness, and difficulty to perform daily activities, decreased significantly at midpoint and final point over baseline in the blueberry group. In the placebo group, there was no change in the WOMAC total score. Increased normal pace walking cadence and velocity were observed at final point over baseline in both groups. Normal paced step and stride length for both limbs increased at midpoint over baseline and continued to increase at final point in the blueberry group. Normal waking pace single support percentage for both limbs increased at final point over baseline, while double support percentage for both limbs decreased in the blueberry group. For the inflammation changes, no significant changes were observed in plasma concentrations of tumor necrosis factor (TNF)-α, interleukin(IL)-1β, IL-6, IL-10, IL-13, matrix metalloproteinases (MMP)-3, and MMP-13, in the blueberry group. However, significant increases in TNF-α and IL-1β were noted at midpoint over baseline in the placebo group. Weight and BMI were significantly increased in the placebo group, while individuals in the blueberry group maintained their weight and BMI during the

study period. Systolic and diastolic blood pressure decreased significantly in the blueberry group at midpoint over baseline and at final point over baseline, while there were no changes observed in the placebo group. However, there were no significant differences between the blueberry and the placebo group in regard to the WOMAC total and sub-group scores, gait parameters, and inflammatory and anti-inflammatory biomarkers, weight, BMI, and blood pressure at any time point.

Conclusions: The findings of this study suggest that daily incorporation of whole blueberries may reduce pain, stiffness, and difficulty to perform daily activities, while improving gait performance, and would therefore improve quality of life in individuals with symptomatic knee OA.

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INTRODUCTION

Osteoarthritis (OA) can be defined by symptoms such as pain and decreased flexibility. It is typically evaluated or classified through radiographic X-ray (Felson et al., 2000). It often affects joint cartilage and/or underlying bones, involving the articular surfaces of synovial joints (Escott-Stump, 2012). Symptomatic OA is defined as the presence of radiographic OA in combination with symptoms attributable to OA (Felson et al., 2000). The primary symptoms of OA are joint pain, aching, and stiffness. OA is the most common joint disorder in the world and is the most frequent cause for walking-related disability among older adults in the United States (Felson et al., 2000). It has been identified as an inflammatory disease of synovial joints and due to the symptoms and pathogenesis related structural changes of OA, it impacts one's gait performance, therefore limiting physical activities (Stefanik et al., 2016).

According to the National Health and Nutrition Examination Survey III (NHANES III), the prevalence of radiographic knee OA for those 60 years old and older is 37%. In addition, the prevalence of symptomatic knee OA among the same age group is 12% (Dillon, Rasch, Gu, & Hirsch, 2006). The overall number of adults in the US who are afflicted with OA is on the rise, from 21 million in 1995 to 27 million individuals in 2005 (Lawrence et al., 2008). Based on findings from the Johnston County study, the

lifetime risk of developing symptomatic knee OA has been estimated at 40% for men and 47% for women (Murphy et al., 2008).

Current treatments for OA include pharmacologic, non-pharmacologic, and surgical intervention. Common pharmacological agents used include acetaminophen and other anti-inflammatory medications. For refractory pain, opioids are used in some cases (Nelson, Aleen, Golightly, Goode, & Jordan, 2014). Use, especially long-term, of these pharmacological agents have potential adverse effects that can lead to serious consequences, that may include gastrointestinal bleeding with long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) (Bjarnason, Hayllar, Macpherson, & Russell, 1993) and adverse cardiac effects associated with long-term use of cyclooxygenase-2 (COX-2) inhibitors (Borer & Simon, 2005). Current pharmacological interventions only have a palliative benefit relieving the symptoms of OA and while not treating the underlining problem of cartilage breakdown. Therefore, alternative, natural, and effective methods of reducing symptoms along with promoting healing and repair of cartilage tissue are warranted.

There is a growing body of research demonstrating a relationship between increased polyphenol intake and its protective benefit in reducing risk for chronic human diseases, such as cardiovascular disease, hypertension, cancers, diabetes, certain infectious diseases, and osteoarthritis (Pandey & Rizvi, 2009). The proposed mechanism by which polyphenols reduce the risks of chronic human disease involves their ability to accept electrons from free radicals, thereby disrupting chain oxidation reactions, and increasing cellular antioxidative capacity (Clifford, 1999). Numerous *in vitro* studies and

animal studies, and a small number of clinical trials, have demonstrated polyphenols' chondroprotective and anti-inflammatory effects (Dave et al., 2008; Huang et al., 2010; Csaki, Mobasheri, & Shakibaei, 2009; Ahmed et al., 2005; Rasheed, Akhtar, & Haqqi, 2010; Wang, Gao, Chen, Li, & Tian, 2012; Belcaro et al., 2010; Panahi et al., 2016).

Blueberries are consumed worldwide. Besides their pleasing taste, they are significant source of polyphenols (USDA Database, 2015). The major polyphenols found in blueberries are anthocyanins, a type of flavonoid. Both in vitro and in vivo studies on anthocyanin effects in joint tissue have shown an anti-inflammatory benefit (Jones, 2016). Studies have shown that flavonoids increase the cartilage anabolic activity by enhancing certain factors such as insulin growth factor-1 (IGF-1), osteocalcin, bone morphogenetic protein (BMP) etc. (Trzeciakiewicz, Habauzit, & Horcajada, 2009; Horcajada & Offord, 2011). A few clinical trials have also shown that blueberries have a potential anti-inflammatory effect (McAnulty et al., 2011; Mcleay et al., 2012) and could may improve functionality in older adults (Schrager, Hilton, Gould, & Kelly, 2015). However, there have been no studies with blueberries investigating their effects on pain reduction, functionality improvement, and inflammation in individuals with OA. This study will examine the effects of daily whole blueberry consumption in individuals with symptomatic OA.

Hypothesis and Specific Aims

Hypothesis

Daily incorporation of 40 grams of whole freeze-dried blueberry powder into the diet of individuals with symptomatic knee OA will reduce pain associated with OA, improve gait, and decrease inflammation.

Specific Aims

Aim 1. To examine the effects of freeze dried whole blueberry in comparison to a placebo powder on knee pain in men and women with symptomatic knee OA.

Aim 2. To examine the effects of freeze dried whole blueberry in comparison to a placebo powder in gait performance in men and women with symptomatic knee OA.

Aim 3. To evaluate the effect of freeze dried whole blueberry when contrasted with a placebo powder on selective plasma inflammatory biomarkers and modulators to include IL-1β, IL-6, TNF- α, MMP-3 and MMP-13, and anti-inflammatory biomarkers, IL-10 and IL-13.

CHAPTER II

REVIEW OF LITERATURE

Osteoarthritis

OA is the most common joint disorder in the world and is the most frequent cause of walking-related disability among older adults in the United States. OA can be defined by symptoms that include pain, aching, stiffness and decreased flexibility and is typically evaluated or classified through radiographic X-ray (Felson et al., 2000). It often affects joint cartilage and/or underlying bones, involving the articular surfaces of synovial joints (Escott-Stump, 2012). It can affect any joint, but mostly affects knees, hips, lower back, neck, small joints of the fingers, and the base of the thumb and big toe (Arthritis Foundation). Symptomatic OA is defined as the presence of radiographic OA in combination with symptoms attributable to OA (Felson et al., 2000). The complete etiology of OA is unknown. The pathology often involves damage and loss of articular cartilage, attrition of subarticular bone, abnormal bone remodeling, growth of osteophytes, and in some cases synovial distension and inflammation (Arden and Nevitt, 2006). Risk factors that contribute to the development of OA are diverse, and typically can be categorized into two groups: local joint specific risk factors and systemic risk factors. Some of the risk factors cannot be changed or altered such as age, sex, genetics, but other risk factors such as obesity, physical activity, and nutrition can be altered to slow down the development of OA (Suri , Morgenroth, & Hunter, 2012).

According to the NHANES III, the prevalence of radiographic knee OA for those 60 years old and older is 37%. In addition, the prevalence of symptomatic OA among the same age group is 12% (Dillon et al., 2006). From the Framingham study, the prevalence in individuals who are 45 years old and older affected by radiographic knee OA is 19% and affected by symptomatic knee OA is 7% (Felson et al., 1987). The overall number of adults in the US who are afflicted with OA is on the rise, from 21 million in 1995 to 27 million individuals in 2005 (Lawrence et al., 2008). The years living with disability (measurement of the burden of disease) for the global population from hip and knee OA increased from 10.5 million in 1990 (0.42% of total disability-adjusted life years) to 17.1 million in 2010 (0.69% of total disability-adjusted life years). Based on Lawrence et al., by 2020, it is predicted that 25% of adults, approximately 50 million people in the US, will be afflicted by OA (2008).

Economic Burden

The Global Burden of Disease study has reported that out of 291 conditions studied, hip and knee OA were ranked as the 11th highest contributor to global disability in 2010 (Cross et al., 2014). The average annual cost to an individual who lives with OA (knee and/or hip) is approximately $10,000 (Gupta, Hawker, Laporte, Croxford, & Coyte, 2005). According to the Center for Disease Control and Prevention (CDC), the total costs attributable to arthritis and other rheumatic conditions (AORC) in the United States was approximately $128 billion in 2015 (CDC, 2015). Based on these numbers, knee OA is one of the leading causes of global disability and creates a huge economic burden. With

aging and a rising incidence of obesity in the world's population, there will be continuously increasing demand for treating knee OA (Cross et al., 2014).

Pathophysiology

Features of OA typically involve degeneration or progressive loss of the structure and function of articular cartilage. The complete mechanism of cartilage degradation is still unclear, but the progression usually involves an imbalance between cartilage formation and breakdown. The cause of OA has been proposed to be related to a complex interplay of genetics, environmental, metabolic, and biochemical factors (Sandell & Aigner, 2001). During the degradative process, matrix-degrading enzymes are overexpressed. This results in loss of collagens and proteoglycans, which are important components of cartilage. In response to the degradation, the cartilage cells (chondrocytes) compensate through increased proliferation and synthesis of matrix molecules in an attempt to enhance the failing structure of the cartilage matrix. Inflammatory cytokines inhibit the synthesis of matrix metalloproteinase (MMP) inhibitors, leading to an increase in MMPs and a corresponding increase in cartilage breakdown (Martel-Pelletier et al., 2016). The inflammatory cytokines include IL-1β, IL-6, IL-7, IL-8, and TNF-α. These cytokines, also involved in nerve-injury/inflammation-induced central sensitization in OA, are related to the development of contralateral hyperalgesia, which can affect mobility (Zhang & An, 2007).

During matrix synthesis, anabolic factors are produced, such as insulin-like growth factor-I (IGF-I) and transforming growth factor – beta (TGF-β). Matrix synthesis and degradation occur simultaneously, however, the progression of OA is caused by the

breakdown of cartilage that far exceeds the formation response to repair and build cartilage. Overall, this leads to the loss of cartilage tissue (Wollheim, 1999).

Risk Factors

Factors that contribute to the development of knee OA include systemic risk factors and local biomechanical risk factors. The systemic risk factors are age, gender, race/ethnicity, genetics, obesity, osteoporosis, bone density, and nutrition. The local biomechanical risk factors are joint injury, certain occupations, physical activities, limb-length inequality, neuromuscular factors, bone characteristics, and joint space (Suri et al., 2012).

Age is consistently a strong risk factor for the prevalence and incidence of knee OA. As age increases, the risk of developing knee OA increases (Lawrence et al., 2008). Female gender is associated with higher prevalence of symptomatic knee, hip and hand OA and greater rate of progression (Srikanth et al., 2005). A higher prevalence has been observed among African Americans – heritability influences radiographic OA (Dillon et al., 2006), and certain genotypes may influence the risk of OA (Kerkhof et al., 2010). Obese or overweight individuals have nearly 3 times the risk of incident knee OA compared with those who have normal weight (Blagojevic, Jinks, Jeffery, & Jordan, 2010). High bone mineral density is associated with a 2.3 – 2.9 greater odd of incidence of radiographic knee OA according to the population based multi-center osteoarthritis study (Nevitt et al., 2010). Studies have also shown that nutrition has an impact on the development of OA and is one of the modifiable risk factors. Intake of Vit D, Vit C, and Vit K maybe associated with lower risk or slower progression of OA (Breijawi et al.,

2009; Bergink et al., 2009; McAlindon et al., 1996; Peregoy and Wilder, 2011; Oka et al., 2009; Neogi, Felson, Sarno, & Booth, 2008).

For local specific risk factors, joint injury is a potent risk factor for future OA. Some evidence supports a greater risk of knee OA with the specific activities of excessive kneeling, squatting, climbing steps, prolonged standing, and lifting. The Framingham study showed that recreational physical activity does not increase the risk of radiographic OA (Felson et al., 1991). However, high intensity exercise increases the risk of OA that is further confounded with increased risk of joint injury. Limb-length inequality can be a risk factor due to the changes in load during weight bearing or compensatory gait patterns. Certain muscle weakness may increase risk of knee OA (Hunter & Eckstein, 2009). Studies have shown that strengthening the knee extensor can help protect against symptomatic OA in women (Lane, Hochberg, Pressman, Scott, & Nevitt, 1999). Also, bone and joint properties including space and alignment, and anatomic relationships play an important role in the development of OA associated with weight bearing (Baker-LePain and Lane, 2010).

One major symptom of osteoarthritis is pain. Based on a study by Bedson and Croft, the prevalence of knee pain in patients with radiographic knee OA ranged from 15% to 81% (Bedson & Croft, 2008). As more pain research has been done over the past years, the mechanism of OA pain has become better understood and are thought to be involved at three different levels. The first level is the local pathological process in the

OA joint leading to pain; the second level is a neuronal mechanism and alterations of pain processing involved in OA pain; and the third level is general factors such as genetic and metabolic factors that may have an effect on OA pain (Eitner, Hofmann, & Schaible, 2017).

Local Pathological Process

As OA progresses, the joint pathological process may change. Along with progressive destruction of cartilage, subchondral bone sclerosis, osteophytes, inflammation such as synovitis, and bone marrow lesions have all been shown as a part of the OA progression (Chen et al., 2017). Some studies reported associations between structural damage and pain. Neogi *et al*. found that knee pain occurred in a higher proportion of OA patients with Kellgren/Lawrence (K/L) grade 4 compared to OA patients with K/L grades 2 and 3 (Neogi et al., 2009). Eckstein *et al*. reported that knees with frequent pain displayed greater rates of medical cartilage loss in a longitudinal study (2011). As reported by Kaukinen *et al*., osteophytes were strongly associated with knee pain (Kaukinen et al., 2016). Zhang *et al*. reported that pain in knee OA fluctuates with changes in bone marrow lesions and synovitis. Pain reduces in intensity and frequency when bone marrow lesions become small, while worsening of the synovitis and effusions are associated with increased risk of more frequent and severe pain (Zhang et al., 2011). A positive relationship between inflammatory changes and joint pain were also shown in MRI studies (de Lange-Brokaar et al., 2015; Yusup et al., 2015).

Neuronal Mechanisms

Eitner, Hofmann, and Schaible (2017) observed that patients with knee OA experience pain in joints not only during activities, but also under resting conditions.

Eitner et al. (2017) also found that patients with higher synovitis scores suffered from more severe pain. Pain is also an expression of a highly sensitized nociceptive system of the joint. Neuronal changes at several levels were identified in OA, including peripheral sensitization, central sensitization, reduced descending inhibition, and atrophy of cortical areas (Eitner et al., 2017). These changes are involved in pain mechanisms in OA patients. The mediators and mechanisms involved in nociceptive pain will be discussed in the Osteoarthritis and Inflammation section.

General Factors

OA may be the result of numerous pathogenic factors. Some of the risk factors may represent cofactors or comorbidities that have a direct or indirect impact on the sensation of pain. Numerous patients with OA are also obese, which may be a significant comorbidity for many reasons. High body weight will put a greater load on joints, as adipose tissue has the capacity to release cytokines, a higher amount of adipose tissue may release more proinflammatory cytokines, creating a chronic low-grade systematic inflammation. Adipose issues may contain high number of resident macrophages, which can produce cytokines that include IL-1β, TNF-α, IL-6, leptin, and adiponectin along with others. Some proinflammatory cytokines regulate metalloproteinases, which are involved in cartilage degradation. Diabetes Mellitus may be an important factor impacting the severity of OA pain. In a study that investigated whether the presence of obesity and diabetes mellitus influence pain intensity at the end stage of OA, it was found

that diabetic patients exhibited significantly increased pain intensity during loading the joint, sitting, and lying down (Eitner et al., 2017). The reason why diabetic patients experience more intense OA pain is suspected to be associated with more severe synovitis and higher concentrations of IL-6, which can induce a long-lasting sensitization of joint nociceptors (King & Rosenthal, 2015).

Some other factors that may play a role in OA and OA pain include psychological and socioeconomic factors and genetic factors. A study has found that individuals who have high optimism are associated with lower self-reported pain, lower sensitivity to mechanical, pressure, and thermal pain. On the other hand, individuals with low optimism are associated with high sensitivity to mechanical pressure, thermal stimuli, and significant central sensitization to mechanical and thermal stimuli (Cruz-Almeida et al., 2013). Also, Cleveland et al. have found that individuals with knee OA who are at the highest risk of developing disability and pain are those who have a lower socioeconomic status (Cleveland et al., 2013). Genetics play an important role in various aspects of OA generation such as bone morphogenetic proteins, apoptosis and mitochondrial damage, extracellular matrix components, inflammation and immune responses, cartilage-degrading enzymes, and pain (Thakur, Dawes, & McMahon, 2013). Warner and Valdes have examined about 400 genetic markers in the genome of patients with OA and have identified single nucleotide polymorphisms that are associated with pain, implicating a genetic contribution to pain sensitivity (Warner & Valdes, 2016).

In a clinical setting, pain and other symptoms of OA, such as stiffness, are usually evaluated using questionnaires such as WOMAC and/or the Knee Injury and Osteoarthritis Outcome Score (KOOS) (Eitner et al., 2017).

Osteoarthritis and Gait

Biomechanics of Human Walking

Walking is one of the most basic methods of human displacement and the purpose of walking, from a mechanics standpoint, is to transfer body weight safely and effectively on a flat or uneven terrain. During walking, the following steps are carried out: maintaining an upright and balanced body posture; control of the trajectory of the lower limbs to keep a safe distance of the feet with the floor and perform gentle contact with the ground; production of mechanical energy to control the speed in the direction of movement; and, absorption of shock forces to ensure the balance of the body (Bertomeu-Motos, 2016). A gait cycle is composed of all phases of walking, and is defined by the time interval between two successive occurrences of one of the repetitive events of the walking maneuver.

The basic gait cycle for walking is shown in Figure 1.0. A gait cycle is divided into two main phases, stance phase and swing phase. The stance phase is described as both feet being on the ground, and the swing phase is described as only one foot being on the ground. The stance phase is further divided into loading response (foot flat), mid stance, terminal stance (heel off), and pre-swing (toe off). Using the right foot as an example, the right stance phase begins with the heel contact of the right foot and ends

with the toe off of the same foot. The swing phase is further divided into initial swing, mid swing, and terminal swing phases. The right swing phase begins with the toe off of the right foot off the ground and ends with the heel contact with the ground of the same foot. The stance phase normally makes up 60% of a gait cycle and the swing phase makes up 40% of a gait cycle. A gait cycle also includes two support components, which are single support and double support. The single support is defined as only one foot in contact with the floor and the double support is defined as both feet in contact with the floor. Usually, the single support makes of 80% of a gait cycle (40% for each limb) and the double support makes up 20%. During an increased walking speed, the percentage of total time (given one complete gait cycle) involved in the stance phase decreases, while time involved in the swing phase increases, and double support decreases (Bertomeu-Motos, 2016; Perry & Davids, 1992).

Variables used in Gait Analysis

The variables used for gait analysis include spatial (distance) and temporal (time) variables. The spatial variables include step length, stride length, width of walking base, and foot angle (degree of toe out or angle gait). Step length is the distance between corresponding successive points of heel contact of the opposite feet. In a normal gait, right step length is equal to left step length. Stride length is the distance between successive points of heel contact of the same foot. In a normal gait situation, the stride length should be a double of the step length. Walking base is the side-to-side distance between the line of the two feet, also known as “stride width”. The degree of toe out represents the angle of foot placement and may be found by measuring the angle formed

by each foot's line progression and a line intersecting the center of the heel and the second toe. The degree of toe out decreases as the speed of walking increases in a normal gait situation (Whittle, 1993).

Time variables include step, stride, stance, swing, single support, double support time, cadence, and velocity. Step time refers to the amount of time spent during a single step. It is the time between heel strike of one leg and heel strike of the contra-lateral leg. The stride time refers to the amount of time it takes to complete one stride. Stride duration and gait cycle duration are the same. Stance time is the amount of time that passes during the stance phase of one extremity in a gait cycle. It includes single support and double support. Swing time is the amount of time that passes during the swing phase of one extremity in a gait cycle. The swing time can be calculated based on stance time and stride time. If the stride time of a gait cycle is 1 second, the stance time is 0.6 seconds, then the swing time is 0.4 seconds. Single support time is the amount of time that passes during the period when only one extremity is on the supporting surface in a gait cycle. And, the double support time is the amount of time that a person spends with both feet on the ground during one gait cycle (Perry & Davids, 1992). Cadence is the number of steps per unit time; a normal cadence is in between 100 to 115 steps per minute. The velocity is defined as distance covered by the body in unit time and it is usually measured in meters per second (Whittle, 1993).

Gait Characteristics of Patients with Knee OA

Individuals with knee OA experience pain, stiffness, and decreased range of motion of the joints, which can significantly limit one's ability to perform daily activities

such as standing, walking, climbing stairs, and rising from a chair or bed. The inability or limitations in performing daily activities may also lead to loss of functional independence. In order to better understand how OA impacts gait, Kaufman *et al*., measured knee kinematics and kinetics of 139 patients with Grade II knee OA compared to healthy individuals with normal gait. Gait was measured during level walking, stair ascent and stair descent. The results showed that individuals with OA walked slower (lower velocity, $P < 0.01$) compared to healthy individuals, and had a significantly reduced internal knee extensor moment during stair ascent ($P = 0.02$), which reflects compensation behavior to reduce the knee joint loading. Kaufman et al., also observed that female subjects had significantly greater knee flexion ($P = 0.0001$) during the stance phase and a greater knee extensor moment ($P = 0.001$) (Kaufman, Hughes, Morrey, Morrey, & An, 2001). A number of studies have also reported slower walking speed in patients with OA compared to non-OA individuals, and slower walking speed in severe OA patients compared to moderate OA patients (Astephen, Deluzio, Caldwell, & Dunbar, 2007; Mills, Hunt & Ferber, 2013). Some other gait changes have been observed in patients with OA, such as single support phase (percentage of gait cycle), stride length, and cadence.

In a multicenter OA study, the GAITRite® system was used to evaluate gait performance and investigate the relation of step length to the sex-specific prevalence and worsening of MRI-detected structural damage in patellofemoral joint (PFJ) among older women and men with or at risk for knee OA. The study found that, in women, individuals with a shorter step length have higher odds of cartilage damage and bone marrow lesions

(Stefanik et al., 2016). In one study of 125 individuals with bilateral medial compartment symptomatic knee OA, researchers found a strong correlation between single support phase and WOMAC-pain, WOMAC-function, Short form health survey-36 pain sub category, velocity and step length (Debi et al., 2011). One large scale study (n = 2911) investigated a novel classification method for knee OA based on spatiotemporal gait analysis and found that using stride length and cadence as classification parameters of OA severity correlates with radiographic evaluation, the level of pain, function, and number of total knee replacements (Elbaz et al., 2014). Based on current evidence, gait changes can be used in monitoring OA progress and severity.

Gait performance can be analyzed using the GAITRite® system, a portable 10-meter electronic walkway, which has been demonstrated as a valid and reliable tool used to determine a myriad of spatiotemporal gait parameters (Salaffi et al., 2003).

Osteoarthritis and Inflammation

Role of Inflammation in the Pathogenesis of OA

With the progression of molecular biology, researchers were able to understand OA from a cellular and a molecular level, helping to recognize OA as an inflammatory disease. Many studies suggest that inflammation occurs at the earliest stage of OA and contributes to the progression of OA. In one study, serial arthroscopies performed on knees with symptomatic but pre-radiographic OA found an association between the presence of synovitis and the future development of medical cartilage loss (Ayral, Pickering, Woodworth, Mackillop, Dougados, 2005).

Studies using magnetic resonance imaging (MRI) have also suggested an association between presence of synovitis and OA progression (Krasnokutsky et al., 2011; Roemer et al, 2011). In one study of 70 synovial tissues with different severities of radiographic OA, severe synovial inflammation was observed in 31% of patients, and synovial inflammation was present in many subjects with minimal radiographic disease (Haywood et al., 2003). Besides local inflammation, such as synovitis, systemic low-grade inflammation was also a driver of ongoing joint degeneration. The development of chronic inflammation in OA following joint injury or overuse can be described as a vicious, self-perpetuating cycle of local tissue damage, inflammation, and repair (Scanzello, Plaas, & Crow, 2008). At the cellular level, following joint injury or overuse, tissue damage results in the production of damage-associated molecular patterns (DAMPs), including cartilage extracellular matrix breakdown and intracellular signaling to increase macrophages and to induce the production of inflammatory mediators. Inflammatory induced angiogenesis and increased vascular permeability under stress results in the subsequent influx of plasma proteins also capable of functioning as DAMPs. The production of inflammatory mediators promotes further cartilage degradation, forming a vicious cycle of the progression of OA.

Even though OA unlike rheumatoid arthritis is not associated with a robust adaptive immune response, the innate immune response also plays a central role in the progression of OA. Post tissue damage, the pattern-recognition receptors also recognize multiple endogenous “danger signals,” known as DAMPs. The DAMPs signal to the immune system to start a protective response to either combat infection or initiate repair

processes. The DAMPs associated with OA include cartilage extracellular matrix (ECM) derived components, plasma proteins, intracellular alarmins, and crystals. Homandberg and Hui (1996) suggested that ECM breakdown products could promote inflammation and cartilage loss. It was observed that fragments derived from the breakdown of fibronectin injected into knees of adolescent rabbits, which resulted in cartilage damage (Homandberg, Meyers, & Williams, 1993), demonstrated that fibronectin fragments induce the production of proinflammatory cytokines and MMPs, such as TNF-α, IL-1β and MMP-1 and MMP-3 (Homandberg & Hui, 1996). With tissue damage and inflammation, tissue permeability increases and vascular leakage occurs, providing a pathway for plasma proteins to enter joints especially synovial fluid. An investigation of OA synovial fluid found increased levels of plasma protein and a number of proteins acting as DAMPs in the elicitation of an inflammatory response. Among the observed plasma proteins, Gc-globulin, α1-microglobulin, and α2-macroglobulin, were capable of inducing production of macrophage, then inducing the production of inflammatory cytokines including TNF-α, IL-6, IL-1β, and vascular endothelial growth factor (Sohn et al., 2012). Therefore, in addition to local production of DAMPs associated with joint injury, there appears to be an influx of inflammatory mediators resulting from vascular leakage, further promoting cartilage breakdown.

Intracellular proteins released from stressed, damaged, or necrotic cells can act as a third potential source of DAMPs. These intracellular proteins include high-mobility group box 1 protein (Liu-Bryan &Terkeltaub, 2010) and the S100 family of proteins (Van Lent et al., 2012). In vitro studies demonstrated the ability of these proteins to induce

TLR4-dependent cartilage catabolism through upregulation of catabolic mediators, such as MMP-1, 3, 9, 13 and proinflammatory cytokine IL-6 (Schelbergen et al., 2012). Besides proteins, inorganic crystals including calcium phosphate (BCP) and calcium pyrophosphate dihydrate (CPPD) are also frequently observed in OA synovial fluids and tissues (Rosenthal, 2011).

Numerous studies suggest that calcium-containing crystals promote inflammation through their interaction with the innate immune system. Liu-Bryan, Pritzker, Firestein, and Terkeltaub (2005) found that CPPD was able to induce chondrocyte production of nitric oxide in a Toll-like receptor (TLR)2-dependent manner. Martinon, Pétrilli, Mayor, Tardivel, & Tschopp (2006) also demonstrated that CPPD interacts with macrophages to induce caspase-1-mediated activation and subsequent release of proinflammatory cytokines including IL-1β and IL-18. Pazar et al. found that BCP behaves in a similar manner in driving inflammation (2011). Furthermore, BCP also induces additional inflammatory mediators, including prostaglandins and MMPs (McCarthy and Cheung, 1994). In addition to calcium containing crystals, uric acid is identified as a contributor to inflammatory processes and cartilage degradation in OA. Denoble et al. found a strong association between synovial fluid uric acid levels and radiographic progression of OA (2011).

Cytokines as Mediators in OA

Inflammatory factors including cytokines, chemokines, adipokines, neuropeptides, and lipid inflammatory mediators have been implicated in OA pathogenesis. This review will focus on cytokines, as this group of inflammatory

mediators is the focus of the research. Studies have shown elevated levels of certain cytokines in OA patients. Kaneko et al. observed increased levels of IL-6 and IL-8 in OA serum and synovial fluid (2000) and Sohn *et al*. identified elevated levels of a large repertoire of cytokines in both blood and synovial fluid of patients with OA (Sohn et al., 2012). Furthermore, Scanzello et al. demonstrated elevated levels of synovial fluid IL-15 in early knee OA and revealed a positive correlation between levels of synovial fluid IL-15 with the levels of synovial fluid MMP-1, MMP-3, and IL-6 (2009). Numerous in vitro studies have revealed an overall catabolic regulatory role of cytokines in the OA joint. IL-1β and TNF-α signal the activation of nuclear factor κB (NFκB) and activator protein 1 transcription factors, which can induce autocrine production of IL-1β and TNF-α and the expression of other inflammatory and chrondrolytic mediators, including MMP-1, MMP-9, MMP-13, and IL-6 (Attur, Patel, Patel, Abramson, & Amin, 1998).

IL-1β is considered one of the key cytokines involved in the pathogenesis of OA. It induces inflammatory reactions and catabolic effects impacting the articular cartilage and other components of joints. Patients with OA have an elevated level of IL-1β in synovial fluid, the synovial membrane, cartilage, and the subchondral bone layer (Massicotte et al., 2002; Sohn et al., 2012). Studies have confirmed that IL-1β functions by blocking chondrocytes in the synthesis of ECM components, interfering in the synthesis of the key structural proteins that include type II collagen and aggrecan (Shakibaei et al., 2005; Stöve, Huch, Günther, & Scharf, 2000). In addition to blocking the synthesis of structural proteins, IL-1β affects chondrocytes' synthesis of enzymes from the group of MMPs, mainly MMP-1, MMP-3, and MMP-13, which have destructive

effects on cartilage components (Vincenti and Brinkerhoff, 2002; Meszaros and Malemud, 2012). Chondrocytes subjected to the effect of IL-1β and TNF-α also tend to age more rapidly and respond to induced apoptosis (Ye et al., 2014). Furthermore, IL-1β stimulates the production of reactive oxygen species, leading to the generation of peroxides and hydroxylated radicals. These peroxides and radicals directly damage the articular cartilage via a process that is associated with decreased expression of oxidative enzymes (Afonso et al., 2007).

TNF-α is also considered a key inflammatory cytokine involved in the pathophysiological process of OA (Bodmer, Schneider, & Tschopp, 2002). TNF-α behaves similarly to IL-1β by blocking the chondrocyte synthesis of proteoglycan components and type II collagen (Seguin & Bernier, 2003), and inducing the synthesis of MMP-1, MMP-3, and MMP-13 (Xue, Wang, Liu, & Luo, 2013), which induce chondrocyte death and a disorder in the migration of chondrogenic progenitor cells. This migration strips the cartilage of any possibility of regeneration (Joos, Wildner, Hogrefe, Reichel, & Brenner, 2013). Both TNF-α and IL-1β have been shown to reduce the efficiency of the respiratory chain, leading to decreased ATP production within the mitochondria located in chondrocytes (Lopez-Armada et al., 2006). Additionally, TNF-α is responsible for induced synthesis of IL-6 and IL-8 and the production of other enzymes that are involved in the progression of OA including inducible nitric oxide synthase (iNOS), cox-2, and prostaglandin E_2 synthesis (PGE2) (El Mansouri et al., 2011; Hardy et al., 2002).

IL-6 has been found to play a dual role in protecting and promoting deterioration of cartilage cells. IL-6 is considered a cytokine that strongly activates the immune system and enhances the inflammatory response. However, based on some studies it may be considered an anti-inflammatory factor (Hammacher et al., 1994). In synergy with other cytokines, the effect of IL-6 on joints is to decrease the production of type II collagen while increasing the production of MMPs (Poree et al., 2008). However, while analyzing animal models, it can be observed that IL-6 may have a different effect. Mice lacking the gene for IL-6 exhibited a tendency to develop much more advanced degenerative changes compared to healthy mice (deHooge et al., 2005). In other cases of mice lacking the IL-6 gene, injection of IL-6 reduces the loss of proteoglycans in the acute phase of chronic joint inflammation and induced the formation of osteophytes (Van de Loo et al., 1997).

IL-10 is one of the anti-inflammatory cytokines, and has been shown to have chondroprotective effects in the course of OA. Its ability to protect chondrocytes can be expressed via four different pathways. First, IL-10 is involved in stimulating the synthesis of type II collagen and aggrecan, a cartilage specific proteoglycan core protein. An in vitro study found that following the administration of IL-10, both healthy and OA articular cartilage demonstrated an increase in proteoglycan synthesis (Iannone et al., 2001). Second, IL-10 is responsible for inhibiting the production of MMPs (Wang and Lou, 2001). Third, IL-10 also inhibits apoptosis of chondrocytes. It has been shown that IL-10 activates kinase pathways that induce the production of bone morphogenetic proteins that play critical roles in chondrogenesis (Umulis, O'Connor, & Blair, 2009). Thus, IL-10 induces chondrocyte differentiation and proliferation through the bone

morphogenetic protein pathways. Fourth, IL-10 reduces the effect of TNF-α on synovial fibroblasts in OA patients through a significant reduction in the secretion of cox-2 and PGE_2, thus reducing TNF-α's expression and a decrease in its ability to bind to the surface of fibroblasts (Alaaeddine et al., 1999).

Since IL-10 has a chondroprotective effect, researchers have been investigating which factors could increase the production of IL-10. In one exercise study, participants with the same level severity of OA were divided into two groups, a control group and exercise group. In the exercise group, participants worked the knees affected with OA for 3 hours, while the study group refrained from exercise during the same period of time. Synovial fluid was collected before, during, and post exercise for IL-10 evaluation. Results reported a significant increase of IL-10 in synovial fluid and particular tissue in the exercise group, but no change in the control group (Helmark et al., 2010). This research suggested that exercise might be considered an intervention for OA through its potential effect in increasing IL-10 secretion.

IL-13 is another anti-inflammatory cytokine. Its anti-inflammatory, chondroprotective effects on the cells of immune system, articular cartilage, and synovium have been well-documented (Hart, Ahern, Smith, & Finlay-Jones, 1995; de Waal Malefyt et al., 1993). In one study investigating the effects of IL-13 on OA, synovium samples were collected from 16 patients with OA during a knee endoprosthesis implantation procedure. The samples were stimulated with a proinflammatory lipopolysaccharide (LPS) followed by administration of IL-13. After incubating for 72 hours, binding tests, northern blotting, and ELISA were performed. Results showed that

IL-13 inhibited the synthesis of proinflammatory IL-1β, TNF-α, and MMP-3. It was also observed that in synovium cells, the amount of mRNA for IL-1β was reduced. Synovial fibroblasts showed a reduction in binding between IL-1β and its receptor (Jovanovic et al., 1998). This study demonstrated that IL-13 has the ability to inhibit the proinflammatory effects of TNF-α and IL-1β and protects synovium in OA patients, indicating the potential utility of IL-13 in OA treatment.

Cytokines and OA Pain

It has been shown that proinflammatory cytokines can cause pain via two mechanisms. First, proinflammatory cytokines can induce the production of pain mediators such as prostaglandins, which activate and sensitize nociceptive neurons. Second, they can directly act on nociceptive sensory neurons (Schaible, 2014). A single injection of TNF-α, IL-6, or IL-1β into the normal knee joints led to sensitized nerve fibers involved in innocuous and noxious rotation of the knee joint. The sensitization developed slowly over 1 hour and persisted for hours (Richter et al., 2010; Brenn, Richter, & Schaible, 2007; Ebbinghaus et al., 2012b). Orita *et al*., found in knee OA patients with K/L grade 1 – 4 a positive correlation between TNF-α concentration in the synovial fluid and pain intensity (Orita et al., 2011). Treatment of OA patients with TNF-α antagonist adalimumab resulted in a significant improvement of WOMAC pain, stiffness and function scores, as well as joint swelling (Maksymowych et al., 2012). Diacerein is a low acting agent that inhibits IL-1β synthesis, reduces the IL-1 receptor density, and inhibits the MEK/ERK intracellular cascades leading to a reduction in cytokines and MMPs. Daily oral ingestion of Diacerein in OA patients with moderate to

severe pain resulted in a significant reduction of pain by the WOMAC assessment and Visual Analogue Scale pain score (Kongtharvonskul et al., 2015). A correlation between elevated IL-6 and pain intensity was also found in patients with knee and end-stage OA (Eitner et al., 2017). The research discussed above showed that cytokines play a major role in pain sensitization. Thus, decreasing concentrations of certain proinflammatory cytokines may be considered as an intervention to reduce pain.

MMPs and Inflammation

Increased MMPs expression has been observed in almost every human disease that involves inflammation (Parks, Wilson & Lopez-Boado, 2004). In vitro studies have shown MMPs act broadly in inflammation by regulating barrier function, regulating inflammatory cytokines and chemokines, and generation of chemokine gradients. During the breakdown of epithelial and endothelial barriers, vascular permeability increases to allow the influx of leukocytes into areas of infection and damage. MMPs regulate proteolysis of endothelial cell junctional proteins (Ichikawa et al., 2006; Reijerkerk et al., 2006). Evidence has also shown MMPs can either promote or repress inflammation by direct proteolytic processing of inflammatory cytokines and chemokines (McQuibban et al., 2001; McQuibban et al., 2002). Simultaneously, as discussed earlier, inflammatory cytokines induce the expression of certain MMPs. Due to the involvement of MMPs in the process of inflammation, certain MMPs can be used as biomarkers of inflammation and markers for progression of OA. In experimental OA models, the expression pattern of MMP-13 correlates with the presence of pathological

chondrocytes that undergo hypertrophic differentiation in the early stage of OA development (Kamekura et al., 2005). Another in vitro study has reported that high levels of active MMP-3 expression in human synovial membrane culture and TNF-α stimulated human cartilage. In a cross-sectional study of both OA and RA patients, serum active MMP-3 level was correlated with C-reactive protein and erythrocyte sedimentation rate.

In addition, in patients receiving anti-TNF-α treatment, the serum level of active MMP-3 was significantly reduced compared to baseline level (Sun et al., 2014). Furthermore, in a clinical trial investigating the use of MMP-3 concentration to predict joint space narrowing involving 120 women with unilateral knee OA, participants were randomly assigned to either treatment group, a structure modification with doxycycline, or a placebo group. Joint space narrowing was assessed and MMP-3 levels were collected at baseline, 16 months and 30 months. Subjects in the placebo group whose MMP-3 concentration was in the upper tertile of the baseline distribution showed a 4-fold increase in the odds of progression of joint space narrowing when compared with the lower tertile. MMP-3 concentration was also positively correlated to increased joint space narrowing (Lohmander et al., 2005). This study showed that MMP-3 might be a reliable indicator for joint space narrowing in OA patients.

MMP-13 and MMP-3 have been recognized as the most important MMPs in OA cartilage destruction due to MMP-13's preferential digestion of type II collagen and MMP-3 does not only digest many cartilage extra cellular matrix components such as aggrecan, type IX collagen and link protein but also activates precursors of other MMPs

such as proMMP-1, proMMP-7, proMMP-8, proMMP-9 and proMMP-13 (Takaishi, Kimura, Dalal, Okada, & D'Armiento, 2008).

Current Pharmacological Treatment Options

Current treatments for OA combine nonpharmacologic modalities, pharmacologic agents, and surgical procedures. Nonpharmacological modalities such as exercise, weight control, rest, and heat treatment for relief of pain are used alone or in combination with pharmacologic agents (Nelson et al., 2014).

Common pharmacological agents used are acetaminophen and anti-inflammatory drugs. Acetaminophen is recommended as a first-line in the pharmacologic management of OA. It is a weak inhibitor of the synthesis of prostaglandins. Anti-inflammatory drugs such as NSAIDs and COX-2 inhibitors are used as the next most appropriate treatment agent suggested by a review of multiple OA treatment guidelines. For refractory pain, opioids are recommended. (Nelson et al., 2014). However, pharmacologic agents have potential adverse effects that may lead to serious consequences. A large study (n = 64,839) has shown that long term use of acetaminophen was associated with a 2-fold increased risk of incident hematologic malignancies (Walter, Milano, Brasky, & White, 2011).

Studies have also shown that ingestion of NSAIDs may cause nonspecific colitis in the GI tract, large intestinal ulcers, bleeding, even perforation, and the associated complications of blood loss and protein loss that can lead to more serious health issues (Bjarnason et al., 1993). NSAIDs may also cause relapse of classic inflammatory bowel

disease and contribute to serious complications of diverticular disease (fistula and perforation) (Singh & Triadafilopoulos, 1999). The adverse cardiac effects of long-term use of COX-2 inhibitors have also been observed (Borer & Simon, 2005). A population based nested case-control analysis reported that an increased risk of myocardial infarction was associated with current use of rofecoxib, diclofenac, and ibuprofen (Hippisley-Cox & Coupland, 2005). Furthermore, many adverse effects have been reported associated with the use of opioids including psychological addiction, immunosuppression effects, hormonal changes, opioid-induced hyperalgesia, opioid-induced sedation, and sleep disturbance (Benyamin et al., 2008).

Current pharmacological interventions can only relieve the symptoms of pain and will not treat the underlining problems of cartilage breakdown. Therefore, an alternative, natural, and effective method of reducing pain along with promoting healing and repair of the cartilage tissue would be most useful.

Current Dietary Treatment Options

Current investigation of dietary treatments of OA focuses on certain nutrients, dietary supplements, and dietary pattern. For individuals with OA who are obese, dietary treatment that focuses on weight loss may be one of the most feasible ways to relieve symptoms and potentially slow down the progression of OA.

As discussed in the risk factors of OA section, obesity is one of the risk factors for OA due to both increased physical load to joints and increased concentrations of inflammatory mediators and adipokines (King, March, & Anandacoomarasamy, 2013). In the Framingham cohort, Felson *et al*. noted that weight loss of 5.1 kg over 10 years

decreased the odds of incident knee OA by 50% (Felson, Zhang, Anthony, Naimark, & Anderson, 1992). In a study of 142 obese radiographic evidenced OA patients, each kilogram of weight loss was associated with an approximately 4-unit reduction of knee joint forces and each pound of weight loss was associated with an approximately 4-fold reduction in load on the knee (Messier, Gutekunst, Davis, & DeVita, 2005).

In a clinical trial of obese patients with OA, patients were randomly divided into two energy restricted diet groups: one group was provided with a very low energy diet per day (415 kcal/day) and the other group was provided a low energy diet a day (810 kcal/day). After 8 weeks, both groups were shifted back to a 1200 kcal/day diet for normal food and meal replacement. Both groups had significant improvement in symptoms, but there was no reported significant improvement in pain (Riecke et al., 2010). A meta-analysis, evaluating current evidence regarding the effect of weight reduction in obese OA patients suggested that disability related to OA could be significantly improved when weight was reduced over 5.1%, or at the rate of > 0.24% reduction per week. Clinical efficacy on pain reduction has also been shown with weight reduction (Christensen, Bartels, E. M., Astrup, A., & Bliddal, 2007).

The Action for Health in Diabetes trial prospectively evaluated 2203 obese subjects with knee pain. The patients were randomly assigned to either an intensive lifestyle intervention (ILI) group, which included support for behavior change in diet and physical activity, or were assigned to an education group, where general lifestyle change education was provided. The ILI group had a greater weight loss, more improvement in WOMAC scores, and in physical functions (Foy et al., 2011). Based on these findings,

one of the most effective treatments for obese patients with OA is to lose weight through a dietary method, an exercise method, or a combination of the two. However, weight loss may be first achieved through diet in order to provide a higher likelihood of physical activity, as proper techniques need to be applied during exercise to prevent any injuries or over use of joints (Bliddal, Leeds & Christensen, 2014).

Vitamin D plays an important role in collagen and bone metabolism and is therefore was critical for its potential benefits in OA. As shown in the Framingham Heart Study, individuals with low dietary vitamin D intake and low blood 25-hydroxy vitamin D3 levels were more likely to have progression of established OA but did not increase the risk for OA development (McAlindon et al., 1996). In 1248 subjects from the Rotterdam study on the elderly, the adjusted odds ratio for progression of knee OA was 7.7 for individuals in the lowest versus highest tertile of vitamin D intake (Bergink et al., 2009). However, data from both the Framingham Osteoarthritis Study and the Boston Osteoarthritis of the Knee Study showed no association between 25-hydroxy vitamin D3 level and radiographic worsening of OA (Felson et al., 2007). Also, in a 2-year randomized, placebo-controlled, double-blinded, clinical trial (n=146) studying the effect of vitamin D supplementation on progression of knee pain and cartilage volume loss in patients with symptomatic OA, there was no sufficient elevation in 25-hydroxy vitamin D3 level and no significant reduction in knee pain or cartilage volume in patients in the treatment group when compared to the placebo group (McAlindon et al., 2013). Based on this evidence, though it is suggested that low vitamin status could possibly be related to OA progression and incidence, not all studies confirmed this potential. Further, some

double-blinded randomized controlled trials did not show an effect of vitamin D supplementation in improving the symptoms of OA or slowing the progression of OA.

Inflammation is a major contributor to the development and progression of OA. Any dietary treatment targeting reducing inflammation might be expected to reduce pain and slow the structural deterioration in OA patients. Omega-3 fatty acids that include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are able to partly inhibit a number of aspects of inflammation (Calder, 2013). Hill *et al*. conducted a randomized trial using high dose (4.5 g) vs. low dose (0.45 g) omega-3 fatty acids in patients with knee OA. The study found a significant pain reduction in both groups at 18 months and 24 months (Hill et al., 2016). In a cross-sectional investigation of plasma omega-6 to omega-3 ratio and its relationship to pain, functioning, and distress in adults with knee pain (n=167), a high omega-6 to omega-3 ratio is associated with greater clinical pain/functional limitations, experimental pain sensitivity, and psychosocial distress compared with a low ratio group (Sibille et al., 2018). Current evidence suggests that consumption of omega-3 fatty acids and consideration of a low omega-6 to omega-3 ratio diet may help alleviate pain in patients with OA.

The use of a glucosamine and chondroitin sulfate nutritional supplement to relieve pain in OA has been examined due to its anabolic effects, which include increased chondrocyte proliferation and increased extracellular matrix biosynthesis, and reduction in catabolic events. Though numerous in vitro studies supported these effects, there is a lack of evidence for them in human studies. A large double blind, placebo-controlled 2-year study of 602 patients with knee OA using glucosamine sulfate (1500 mg/day),

chondroitin sulfate (1200 mg/day), or both, found none of the treatments significantly reduced the WOMAC scores compared to placebo (Sawitzke et al., 2008). Later, the same group of investigators found no radiographic evidence that these treatments reduced the progress of OA (Sawitzke et al., 2010). A recent review of clinical trials using glucosamine and chondroitin in OA reported only a few high-quality level I trials exist in the literature on this topic. The effect sizes are generally small and probably not clinically relevant. Even the validity of these results is limited by the high risk of bias introduced in the studies. There is currently no convincing information for the efficacy of glucosamine and chondroitin on OA (Vasiliadis & Tsikopoulos, 2017).

Certain dietary patterns have also been suggested to help manage symptoms of OA and increase physical ability. Clinton et al. (2015) conducted a six-week prospective randomized open labeled study of patients with OA. Participants were randomized to a whole food plant-based diet or to a normal diet as control. The treatment group reported a significantly greater improvement in energy/vitality (assessed by SF-36v2 domain), physical functioning, role physical, and the physical component summary scale compared to the control group. There was also a significant improvement in pain assessed by Visual Analog Scale compared to the control group starting at 2-week time point and on word. The investigators indicated that the potential reasons for the significant improvements in the whole food plant-based diet group may be a result of a change in fatty acid profile, reduction in exposure to inflammatory protein precursors along with the fact that the diet was low in fat and high in fiber, while being less energy dense resulting in lower calorie consumption (Clinton et al., 2015). Data obtained from the Osteoarthritis Initiative of

4470 participants suggested that adherence to the Mediterranean diet is associated with better quality of life, resulting in a lower WOMAC pain and physical activity score, higher Short Form Health Outcome Survey-12 physical composite scale value, and lower Epidemiologic Studies Depression Scale score (Veronese et al., 2016).

Polyphenols and Health

Polyphenols are naturally occurring compounds found in fruits, vegetables, cereals, and certain plant-based products (Pandey & Rizvi, 2009). More than 8,000 polyphenolic compounds have been identified in different plant species (Pandey & Rizvi, 2009). In plants, polyphenols are produced to defend against ultraviolent radiation or aggression by pathogens and contribute to the color and flavor of plant-based foods (Beckman, 2000). There is a growing body of research demonstrating a relationship between increased polyphenol intake and its protective effects in reducing risk of chronic human diseases, such as cardiovascular disease, hypertension, cancers, diabetes, certain infectious diseases, and age-related conditions (Pandey & Rizvi, 2009). The proposed mechanism of polyphenol reduction of risk of chronic human disease involves their ability to accept electrons from free radicals, thereby disrupting chain oxidation reactions, and increasing cellular antioxidative capacity (Clifford, 1999).

Polyphenols and OA in Vitro Studies

Resveratrol, a polyphenolic compound found in grape skin, berries, and peanuts, has been shown in various studies to have chondrocytes protective effects due to its anti-inflammatory and antioxidant properties (Shakibaei, Mobasheri, & Buhrmann, 2011; Dubick & Omaye, 2001). An in vitro study conducted by Shakibaei *et al*. found that

pretreating human primary articular chondrocytes with resveratrol for 4 hours, then treatment with IL-1β and continued treatment of resveratrol led to reduction of IL-1β induced apoptosis and decreased activation of IL-1β caspase-3 activation via ERK1/2 signaling pathway (Shakibaei, Mobasheri, & Buhrmann, 2011). Another in vitro study conducted by Dave *et al*. showed similar results that co-treating human primary chondrocytes, human cartilage explants, or normal bovine chondrocytes with resveratrol and IL-1β resulted in decreased expression and activity of IL-1β induced cox-2, decreased production of PGE_2, decreased IL-1β induced mitochondrial dysfunction, ATP depletion, decreased IL-1β induced apoptosis, and decreased pro-MMP-13 production in cartilage explants (Dave et al., 2008).

Green tea extract rich in polyphenols has been investigated and reported to potentially have anti-diabetic effects (Rizvi, Zaid, Anis, & Mishra, 2005), pain reducing effects in patients with arthritis (Singh, Akhtar, & Haqqi, 2010), and neuro-protective benefits (Rossi, Mazzitelli, Arciello, Capo, & Rotilio, 2008). Huang, Tseng, Lee, Su, & Lee (2010) pretreated human primary OA synovial adherent cells from human synovial tissue with epigallocatechin gallate (EGCG) for 12 hours, a major green tea polyphenol, and then co-treated with IL-1β for 12 hours. Huang et al. (2010) observed a decrease in IL-1β induced cox-2 up-regulation and decreased IL-1β induced PGE_2 and IL-8 production. Andriamanalijaona *et al*. found similar results when pretreating bovine primary articular chondrocytes with EGCG for 24 hours then co-treated with IL-1β. The results showed decreased mRNA level of MMP1, MMP-3, MMP-13, aggrecanase-1,

aggrecanase-2, iNOS, cox-1, and cox-2 and decreased IL-1β induced down-regulation of type II collagen and aggrecan core protein expression (Andriamanalijaona et al., 2005).

Curcumin, a major component of turmeric, also has been efficacious for its anti-inflammatory and antioxidant actions (Aggarwal & Harikumar, 2009). Following pretreatment of human primary chondrocytes with curcumin for 30 minutes and then co-treated with TNF-α for 24 hours, Liacini *et al*. showed decreased TNF-α induced MMP-13 expression (Liacini et al., 2003). Many studies have shown similar results indicating that curcumin pretreated articular chondrocytes or cartilage have decreased MMP-3 and MMP-13 up-regulation (Schulze-Tanzil, Mobasheri, Sendzik, John, & Shakibaei, 2004), decreased IL-6, IL-8 production (Mathy-Hartert et al., 2009), and decreased chondrocyte apoptosis (Csaki et al., 2009).

Nobietin, a citrus polyphenolic compound, has been shown to have anti-inflammatory and antitumor effects (Sato et al., 2002). Ishiwa *et al*. co-treated rabbit synovial fibroblasts with IL-1β and nobiletin and found decreased IL-1β induced pro-MMP-9 mRNA expression and production, decreased PEG_2 production, and decreased proliferation of synovial fibroblasts in growth phase (Ishiwa et al., 2000).

Pomegranate is high in soluble polyphenols that possess antioxidant and anti-inflammatory capabilities (Ahmed et al., 2005). In vitro studies have shown pomegranate's potential to decrease IL-1β induced expression of MMP-1, MMP-3, and MMP-13, decreased DNA binding activity of NF-κB, and decreased proteoglycan released from OA cartilage (Ahmed et al., 2005; Rasheed et al., 2010).

Polyphenols and OA in Vivo Studies

Polyphenols' anti-inflammatory and cartilage protective effects have also been shown in many in vivo studies. In a surgical OA arthritic rabbit model, Wang et al. found that when rabbits with OA ingested of resveratrol (at 20 μmol/kg and 50μmol/kg) for 2 weeks, there was decreased cartilage tissue destruction, decreased loss of matrix proteoglycan content in cartilage, decreased chondrocyte apoptosis, and decreased nitrite oxide in synovial fluid (Wang et al., 2012). EGCG administered to collagen-induced arthritis mice through drinking water slowed disease progression (Dixon, Xie & Sharma, 2005). This disease-modifying effect was associated with a decrease in the inflammatory mediator TNF-α, COX-2, and lower levels of total immunoglobulins (IgG) and type II collagen-specific IgG levels, indicating a reduced inflammatory immune response (Dixon et al., 2005).

In a human randomized controlled study, Belcaro et al. (2010) found increased physical function as evidenced by WOMAC after study participants consumed curcumin-phosphatidylcholine complex (200 mg per day) for 8 months. Belcaro et al. (2010) also found increased walking distance in a treadmill test, along with decreased inflammatory biomarkers in experimental groups. Another randomized controlled clinical trial investigating curcuminoids and their potential to reduce systemic oxidative burden in patients with OA reported its antioxidative effects and its potential in relieving OA symptoms. In the study, 40 patients with mild to moderate knee OA were recruited and randomly assigned to a treatment or a placebo group. Curcuminoids (1500 mg/d divided three times a day) were administrated along with piperine (15 mg/day) to patients in the

treatment group for 6 weeks. Piperine was administrated to increase curcuminoids' bioavailability. There was a significant elevation in serum superoxide dismutase activities, a borderline significant elevation in reduced glutathione concentrations, and a significant reduction in malonedialdehyde concentrations in the curcuminoid group compared with the placebo group. The results suggested that short-term supplementation with curcuminoids attenuates systemic oxidative stress in patients with OA (Panahi et al., 2016). A cross-sectional study of overweight individuals with OA showed that individuals who consumed botanical citrus extracts (*P. amurense* bark and *C. sinensis* peel) for 8 weeks had decreased body weight and blood pressure, decreased joint pain, and had significantly lower high sensitivity C-reactive protein (hs-CRP) levels compared to placebo controls (Oben et al., 2009).

Blueberry

Blueberries are a significant source of polyphenols, especially flavonoids such as anthocyanins, flavan-3-ols, flavones, and flavanols (USDA Database, 2015). The major polyphenols found in blueberries are anthocyanins. Both in vitro and in vivo studies on anthocyanin effects in joint tissue have shown anti-inflammatory effects (Jones, 2016). Previous studies have shown that plant flavonoids reduce circulating inflammatory cytokines and decrease the matrix metalloproteinases MMP-2 and MMP-9, resulting in less bone turnover (Welch & Hardcastle, 2014). Not only do flavonoids slow down catabolic pathways, but flavonoids also increase anabolic activity by enhancing certain

factors such as IGF-1, osteocalcin, and bone morphogenetic protein (BMP) (Trzeciakiewicz et al., 2009; Horcajada & Offord, 2011).

Blueberries and Pain, Gait, and Inflammation

In an in vitro study, Huang, Liu, Wang, Wang, and Li (2014) investigated the inhibitory effect of blueberries' two main anthocyanins (malvidin-3-glucoside and malvidin-3-galactoside) on the inflammatory response in endothelial cells. Huang et al. (2014) found that these two anthocyanins could inhibit TNF-α induced increases of monocyte chemotactic protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) production. In animal studies, researchers found that blueberries reduce pro-inflammatory cytokine production of TNF-α and IL-6 in mouse macrophages by inhibiting NF-κB activation and the mitogen-activated protein kinases (MAPK) pathway (Xie et al., 2011). In human studies, the results of blueberries' anti-inflammatory effects, such as reducing inflammatory specific biomarkers, are not consistent.

Basu et al. administered freeze-dried blueberry powder (50g per day = 350 g of fresh fruit) to 48 individuals with metabolic syndrome for 8 weeks. The powder was provided as a water based drink divided into two beverages per day. At the end of the intervention, they found no significant changes in fasting plasma hs-CRP, IL-6, and adiponectin in the intervention group compared to the control group (Basu et al., 2010). Stull *et al*. investigated blueberries' anti-inflammatory effects in obese men and women with insulin resistance. Freeze-dried blueberry powder was provided to participants, in the quantity of forty five grams per day, in an intervention group for 6 weeks with the

powder blended into a yogurt and milk beverage. At the end of the study, there was no difference in changes in circulating hs-CRP, TNF-α in the intervention group compared to the control (Stull et al., 2010). However, researchers have observed an increase in an anti-inflammatory biomarker (IL-10) in exercise induced models (McAnulty et al., 2011; Mcleay et al., 2012).

Whole blueberries may not only have anti-inflammatory effects but may also improve functional ability. Schrager and colleagues found that consumption of 2 cups of frozen blueberries a day for 6 weeks improved gait speed and a reduced number of step errors during single task adaptive gait, and gait speed during dual task adaptive gait in older adults (age > 60 years old) (Schrager et al., 2015).

The results from the investigation of whole blueberry consumption and its relationship to gait performance are promising, but no studies have been carried out on a knee osteoarthritis model. Furthermore, current studies have not investigated blueberry consumption in relation to pain. Therefore, this proposed research study investigated whether whole blueberry consumption will decrease certain inflammatory biomarkers, increase certain anti-inflammatory biomarkers, reduce pain, and improve gait performance in individuals with symptomatic knee osteoarthritis.

CHAPTER III

METHODOLOGY

Study Design

The study used a double-blind randomized placebo-controlled pre-test and post-test design procedure. A total of 63 men and women, between the ages of 45 and 79, with self-reported symptomatic osteoarthritis were recruited through the local Denton community, Texas Woman's University, and local orthopedic clinics. Recruited participants agreed to not take any cox-2 inhibitors, chondroitin sulfate, glucosamine sulfate, or glucosamine hydrochloride powder, all of which are known to have anti-inflammatory effects and/or influence the symptoms of knee pain. In addition, participants who were recruited agreed to not consume any blueberry products or blueberries during the study.

Eligible men and women were randomly assigned to one of two groups, a treatment group (n = 33) or placebo group (n = 30). The treatment group received 40 g of freeze dried whole blueberry daily. The powder was packaged in 20 g packets, with participants in the treatment group requested to consume 2 packets per day. The placebo group was also asked to consume 40 g of control powder daily, divided into 20 g packages, consumed twice a day. The placebo powder was constituted of maltodextrins and fibers to mimic the carbohydrate composition of whole blueberries, but without whole blueberries. Participants in the treatment and control groups were instructed to reconstitute their respective powders in 10 – 12 ounces of water, immediately followed

by consumption. Participants were on the same regimen, either consuming whole blueberry powder or control powder for a period of 4 months. The study protocols were approved by the Institutional Review Board at Texas Woman's University before any clinical work was initiated (see Appendix D).

Recruiting, Inclusion/Exclusion Criteria

Email and Facebook advertisements (see Appendix A) were used to reach out to potential subjects. The initial screening for identification of potential subjects included a short screening questionnaire (see Appendix B) completed by phone. The screening questionnaire included questions on demographic information, smoking history, medical history, current medications/ supplements, special diet, food allergies, and blueberry consumption. The inclusion criteria were men and women aged 45 to 79, experiencing knee pain, and in relatively healthy condition. The exclusion criteria were men and women who smoke more than one pack of cigarettes per day, have uncontrolled diabetes, who were on an insulin regimen that does not allow additional carbohydrate as part of a routine diet, who have congestive heart failure, who have knee replacements on both knees, or those who were using prescribed cox-2 inhibitors, chondroitin sulfate, glucosamine sulfate, glucosamine hydrochloride powder and were not willing to be off these medications/supplements during the study period. Those who were allergic to blueberries were also excluded from participation.

Baseline, Midpoint, and Final Measurements

Qualified subjects were invited to the study site for 3 visits during the 4-month study period, which included a baseline visit, midpoint visit (at 2 months), and final visit

(at 4 months). At the baseline visit, qualified subjects were provided with a written consent form (see Appendix C) and informed on all aspects of the study. Researchers answered any questions and concerns that participants had. Signing the consent form was completely voluntary. After consent forms were signed, research proceeded according to procedure. The procedures in each visit included anthropometric measurements including weight, height, leg length, and blood pressure, along with obtaining fasting blood samples, performing a walking test (gait test), and filling out a WOMAC questionnaire.

Treatment Compliance

Treatment compliance was tracked using a calendar for daily consumption of blueberry or placebo powder. Calendars were provided to participants at baseline, midpoint, and final visits, and participants were instructed to bring the calendar back on the following visits. Participants were also called for follow up to ensure compliance and to address any concerns.

Blood Collection and inflammatory Marker Analysis

Overnight fasting venous blood was obtained with Ethylenediaminetetraacetic acid (EDTA) anticoagulant added at baseline, midpoint, and final visits by a trained phlebotomist. Blood was centrifuged at 3000 g for 10 minutes to separate plasma, which then was aliquoted into collection tubes and stored at -80 °C for subsequent analysis of IL-1β, IL-6, TNF- α, IL-10, IL-13, MMP-3, and MMP-13. Human High Sensitivity T Cell Magnetic Bead Panel Multiplex ELISA kits from Millipore were used to analyze blood biomarkers of inflammation. Two different panels were conducted. One panel was used to analyze IL-1β, IL-6, TNF- α, IL-10, and IL-13 and the other panel was used for

analyzing MMP-3 and MMP-13. Multiplex ELISA kits utilized Luminex technology, allowing multiple results to be analyzed simultaneously from each sample. Luminex color-codes microspheres by manufacturing beads with precise concentrations of two fluorescent dyes. These bead sets of five hundred 5.6 μm polystyrene microspheres or eighty 6.45 μm magnetic microspheres are then coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection body is introduced. After completing this step, the reaction mixture is incubated with Streptavidin-PE conjugate to complete the reaction, and to make the captured analyte visible to the detector. Luminex 200 software was used to analyze the data. Each microsphere was identified, and the concentration of each biomarker was quantified based on the fluorescent signal.

Assessment of Gait, Balance, and Pain

Gait and balance were analyzed using a GAITRite® system, a portable electronic walkway. The 10 meter GAITRite® system was set up and utilized by trained research personnel. Subjects were instructed to walk 3 trials on the walkway at self-elected (usual) speed and instructed to walk another 3 trials on the walkway at the fastest speed that they can walk without running. Twenty seconds of rest/pause were allowed between each trial. An average of the three trials for each gait velocity was recorded for analysis. The GAITRite® system has been validated and used as a reliable tool to assess a myriad of spatio-temporal gait parameters (DeCaria, Montero-Odasso, Wolfe, Chesworth, & Petrella, 2012).

Pain was assessed by using the WOMAC questionnaire (see Appendix E) at baseline, midpoint, and final visits. These questionnaires have been validated and have been used in many research projects studying symptomatic knee osteoarthritis (Salaffi et al., 2003).

Statistical Analysis

A minimum sample size of 50 participants was needed in order to conduct analysis with alpha = 0.5, power = 0.87, and a moderate effect size. Descriptive statistics were calculated for all variables, comprising means, standard deviations, minima, and maxima for all continuous variables. Frequencies and percentages were calculated for all categorical variables. Distributions of the response variables were examined to determine if statistical tests of hypotheses based on the assumption of normality being met, and that parametric testing is appropriate. Extreme outliers were investigated for technical or clerical error. Baseline differences of dependent variables were tested using independent sample *t*-tests. Although dependent variables in each area are related to each other, due to the small sample size, repeated measures ANOVAs were conducted to examine outcome differences of pain, stiffness, difficulties to perform daily activities, gait performance parameters, and inflammation biomarkers between treatments over time at baseline, midpoint, and final. Most variables are normally distributed, and some variables contain outliers. Therefore, the analyses were done on outlier-removed data as well. Covariate analysis, including ANOVA and regression, were conducted to control for baseline difference. The data was analyzed using SPSS 25.0.0.

CHAPTER IV

RESULTS

Section I Demographics

A total of 116 individuals contacted researchers and showed interest in participating in the study. Of the 116 individuals, 103 individuals were able to be reached and screened for study participation. Of those screened, 63 met the criteria and were scheduled for the baseline visit. Over the course of the study, there were 14 individuals who withdrew from the study due to issues such as taste and palatability of treatment, lack of interest, lack of compliance, or conflicts in study visit scheduling. One participant withdrew at the midpoint visit due to taking the treatment incorrectly but was willing to re-start the study after a wash out period. A total of 49 participants completed the study. Demographic data associated with study participants is provided in Table 1.1 and Table 1.2.

For body weight and height, there was no significant difference between treatment groups at baseline ($p = 0.810$, $p = 0.477$). The body weight increased significantly at final point over baseline ($p = 0.028$) and midpoint ($p = 0.016$) in the placebo group, whereas the blueberry group maintained their body weight throughout the study (see Figure 1.1). At baseline, there was no difference in participants' BMI between the two treatment groups. In the blueberry group, participants maintained their BMI throughout the study period, while participants in the placebo group had a significant increase in BMI overall at final point over baseline ($p = 0.001$) and midpoint ($p = 0.016$) (see Figure 1.2). A significant difference of systolic blood pressure between the two treatment groups was

noted at baseline ($p = 0.048$), but not of diastolic blood pressure. In the blueberry group, participants' systolic blood pressure decreased significantly at midpoint over baseline ($p = 0.021$) and remained at the reduced level at final point over baseline ($p = 0.001$) (see Figure 1.3). Also, participants' diastolic blood pressure dropped significantly at final point over baseline ($p = 0.018$) in the blueberry group (see Figure 1.4). There were no significant changes of systolic and diastolic blood pressure in the placebo group throughout the study. There was no significant difference between the blueberry and the placebo group in regards to weight, height, BMI, or systolic and diastolic blood pressure, at any time point.

Section II WOMAC

The WOMAC questionnaire was used to assess pain, stiffness, and difficulty to perform daily activities during the study. There was no difference in baseline WOMAC total, pain, stiffness, and difficulty to perform daily activities between the two treatment groups. The total WOMAC score significantly decreased at midpoint over baseline ($p = 0.018$) and at final point over baseline ($p = 0.022$) for the blueberry group (see Figure 1.5). There was no change in the total WOMAC score in the placebo group throughout the study. In the blueberry group, pain decreased significantly at midpoint over baseline ($p = 0.008$) and continued to decrease at final point over baseline ($p < 0.001$). In the placebo group, pain was significantly reduced at final point as compared to baseline ($p = 0.036$) (see Figure 1.6). For stiffness, in the blueberry group, there was a significant decrease at midpoint over baseline ($p = 0.005$) and at final point over baseline ($p = 0.045$). In the placebo group, there were no significant changes in stiffness from baseline

to midpoint, but a significant decrease in stiffness at final point over baseline was noted ($p = 0.030$) (see Figure 1.7). Significant decreases in difficulty to perform daily activities were observed in the blueberry group for midpoint over baseline ($p = 0.030$) and final point over baseline ($p = 0.024$). There was no significant change of difficulty in performing daily activities in the placebo group throughout the study (see Figure 1.8). There was no difference between the blueberry and placebo group in pain, stiffness, and difficulty to perform daily activities at any time point of the study.

Section III Gait Performance

Gait performance was analyzed using the GAITRite® system, a portable 10-meter electronic walkway. The parameters collected and analyzed included left and right step length, left and right stride length, single support of cycle for the left and right limb, double support of cycle for the left and right limb, normalized velocity, and cadence. Baseline differences were observed only in the double support normal paced walking right limb, not all the other parameters differed at baseline. Normal paced walking cadence increased significantly in both groups at midpoint over baseline ($p < 0.001$ for blueberry; $p = 0.001$ for placebo) and at final point over baseline ($p < 0.001$ for blueberry; $p = 0.002$ for placebo). The fast-paced walking cadence did not change during the study period in the blueberry group, but there was a significant increase at final point over baseline ($p = 0.016$) in the placebo group (see Figure 1.9). Normal paced velocity increased significantly for both groups at midpoint over baseline ($p < 0.001$) and continued to increase significantly at final point over baseline ($p =$ or < 0.001). In the blueberry group, the increase was also significant at final point over midpoint ($p =$

0.016), but not in the placebo group. There was no change in the fast-paced velocity in the blueberry group, while in the placebo group there was a significant increase at final point over midpoint ($p = 0.009$), but not baseline (see Figure 1.10).

In the blueberry group, the normal paced walking step and stride length increased significantly at midpoint over baseline for both limbs ($p < 0.05$) and at final point over both baseline ($p < 0.05$) and midpoint ($p < 0.05$). At the same time, these parameters also increased significantly in the placebo group at midpoint over baseline ($p < 0.05$) and at final point over baseline ($p < 0.05$). There were no changes noted throughout the study in the fast-paced walking step and stride length for both limbs in the blueberry and placebo group (see Table 1.3). In the blueberry group, there was a significant increase in normal paced walking left single support percentage to one gait cycle at midpoint over baseline ($p = 0.001$) and at final point over both baseline ($p < 0.001$) and midpoint ($p = 0.007$) (see Figure 1.11). At the same time, the decrease in the normal paced walking left double support percentage to one gait cycle was noted at midpoint ($p < 0.001$) and final point over baseline ($p < 0.001$) (see Figure 1.12). A similar pattern was also noted in the normal paced right single and double support percentage to one gait cycle in the blueberry group, that single support percentage increased significantly at midpoint ($p < 0.001$) and final point over baseline ($p < 0.001$), while the double support percentage decreased significantly at midpoint over baseline ($p < 0.001$) and at final point over both midpoint ($p = 0.003$) and baseline ($p < 0.001$).

In the placebo group, there was a significant increase in the normal paced walking left single support percentage at midpoint ($p = 0.016$) and final point ($p = 0.037$) over

baseline, but there was no change in the normal paced right single support percentage (see Figure 1.11). For double support for both limbs in the placebo group, there was no change at final point over baseline, but an increase was observed at midpoint over baseline (left $p = 0.0210$; right $p = 0.041$) (see Figure 1.12). There were no changes in fast-paced single and double support percentage to one gait cycle for either limb in both groups at final point over baseline (see Figure 1.13 and 1.14). Changes at final point over midpoint were noted in the placebo group in fast paced left single support percentage ($p = 0.005$), left double support percentage ($p = 0.005$), and right double support percentage ($p = 0.015$). A baseline difference was noted on the normal paced walking double support percentage to one gait cycle only in the right limb between the two groups. There was no difference at any time point between the two treatment groups in all other gait parameters.

Section IV Inflammation

The inflammatory biomarkers analyzed were IL-6, TNF-α, and IL-1β. Also, the anti-inflammatory biomarkers IL-10 and IL-13, and inflammatory modulators MMP-3 and MMP-13 were analyzed. There were no changes in concentration of inflammatory biomarkers, in the blueberry group as the plasma concentration of TNF-α, IL-1β, and IL-6 remained steady over the study period. In the placebo group, there was a significant increase in the plasma concentration of TNF-α at midpoint over baseline ($p = 0.043$) but decreased at the final point over midpoint ($p = 0.046$). There was also an increase in IL-1β at midpoint over baseline ($p = 0.033$) with a decrease at final point over midpoint ($p = 0.016$), and overall stayed the same for final point over baseline. IL-6 concentration

stayed consistent over the study periods in the placebo group (see Figure 1.15). For the anti-inflammatory biomarkers, the plasma concentration of IL-10 and IL-13 stayed the same over the study period for both treatment groups. However, there was an overall increase in concentration of IL-13 at final point over baseline (+0.30 pg/ml) in the blueberry group, while there was an overall decrease in the placebo group at final point over baseline (-0.46 pg/ml) (see Figure 1.16). The concentration of MMP-3 and MMP-13 in the blueberry group showed a decrease at midpoint over baseline and continued to decrease at final point, but the decreases at both midpoint and final point over baseline did not reach significance (see Figure 1.17 and 1.18). The plasma concentration of MMP-13 stayed the same over the study period in the placebo group (see Figure 1.18). The MMP-3 concentration decreased at midpoint over baseline ($p = 0.020$) but rebounded at the final point (see Figure 1.17). There was no difference at any time point between the two treatment groups in IL-6, TNF-α, IL-1β, IL-10, IL-13, MMP-3, and MMP-13.

CHAPTER V

DISCUSSION

Blueberry is a widely consumed berries that along with its pleasing taste, is also a good source of plant polyphenols. Investigations into blueberries and their effects on bone and joint health have been conducted, with most studies were done in vitro or in animals. Most clinical trials conducted examining blueberry's bone protective effects have only studied one subclass of polyphenols in blueberry typically the anthocyanins. There have been no human clinical trials examining whole blueberry and their effect on pain, functionality, and inflammation in individuals with osteoarthritis. This was a 4-month randomized controlled trial using whole freeze-dried blueberry powder in comparison with a placebo powder control to investigate if whole freeze-dried blueberry powder effects pain, functionality, and inflammation by analyzing pain, stiffness, difficulty to perform daily activities, and inflammatory and anti-inflammatory biomarkers.

The findings demonstrated that freeze-dried whole blueberry powder consumption for a period of 4 months results in a reduction in pain, stiffness, and difficulty to perform daily activities, improved normal walking paced gait performance, and positively impacted certain inflammatory and anti-inflammatory biomarkers. A growing body of research has demonstrated a positive relationship between increased polyphenol intake and its protective effects in reducing chronic human disease risk. The positive effects are attributed to its antioxidant properties and potential anabolic effects in certain cells. Both in vitro and in vivo studies have investigated polyphenols' protective effects on

osteoarthritis. Polyphenols from green tea extract, turmeric, red wine, citrus fruits, and pomegranate have been extensively investigated with many studies showing some level of anti-inflammatory and cartilage protective polyphenol effect.

Results of a WOMAC questionnaire identified decreased pain, stiffness, and difficulty to perform daily activities in the blueberry group after consuming freeze-dried whole blueberry for 60 days (midpoint), with the effects continuing to 120 days (final point). There was a significant decrease in WOMAC total score at both mid- and final-point over baseline for the blueberry group, but no change in the placebo group. These results are encouraging as pain and stiffness are major symptoms of individuals suffering from OA, limiting functional ability therefore lowering quality of life. However, improvements during the blueberry treatment were not significantly different from the change seen in the placebo treatment group at any time point. In another randomized double-blind crossover study investigating the efficacy of a tart cherry juice blend in treatment of knee OA, investigators observed a similar WOMAC scores result that indicated a significant reduction after the cherry juice treatment but not after the placebo treatment; the differences between treatments were not significant (Schumacher et al., 2013). It is anticipated that decreased pain, stiffness, and difficulty to perform daily activity observed in the placebo group was attributable to a placebo effect.

Gait performance was measured using indicators of step length left and right, stride length left and right, single support of cycle left and right, double support of cycle left and right, normalized velocity, and cadence. There was significant improvement in normal paced walking gait performance in the blueberry group, indicated by increased

cadence, velocity, step and stride length for both limbs, increased single support percentage to one gait cycle and decreased double support percentage to one gait cycle for both limbs. The improvement happened as early as 60 days and continued to improve through 120 days. Stride length and cadence are correlated with classification of knee OA severity. Lower stride length and cadence correlated with higher grade OA classified by the Kellgren and Lawrence evaluation system (Elbaz et al., 2014). Single limb support is strongly correlated with WOMAC pain and function, as well as velocity and step length, which correlates with WOMAC pain and function, but not strongly with single limb support (Debi et al., 2011).

Due to double limb support and single limb support collectively forming one gait cycle, when single limb support percentage to one gait cycle decreases, double limb support percentage to one gait cycle increases. The findings of gait performance in normal walking pace are consistent with the WOMAC score changes. Similarly, no significant differences were observed between the blueberry group and placebo group at any time point throughout the study. Schrager, Hilton, Gould, and Kelly (2015) found similar results in older adults (age > 60 years old). In particular, Schrager et al. (2015) found that consumption of 2 cups of frozen blueberries a day for 6 weeks improved gait speed, reduced the number of step errors during single task adaptive gait, and increased gait speed during dual task adaptive gait.

Interestingly, the fast-paced walking gait performance did not seem to improve in the treatment group. As gait velocity increases, functionality and balance requirements increase to maintain the velocity (Middleton, Fritz, & Lusardi, 2015). Improvement in the

fast walking may require better balance and functionality as compared to normal walking. The treatment somewhat helped improve gait performance, but the improvement may not be significant enough to impact fast paced walking.

TNF-α, IL-1β, and IL-6 are inflammatory cytokines that did not change in the blueberry group throughout the study. In the placebo group, plasma concentration of TNF-α and IL-1β increased significantly from baseline to midpoint but stayed the same from baseline to final point. TNF-α, IL-1β, and IL-6 have been found to increase during the worsening of OA and contribute to the progression of OA. Furthermore, these cytokines induce pain by up regulating pain mediators such as prostaglandins. The anti-inflammatory effects of polyphenol have been studied extensively. Most positive results have been observed in *in vitro* studies and animal studies. Shakibaei, Mobasheri, and Buhrmann showed that pretreatment of human primary articular chondrocytes with resveratrol for 4 hours, followed by treatment with IL-1β and continued treatment of resveratrol results in a reduction of IL-1β induced apoptosis (2011). EGCG administration to collagen-induced arthritic mice through drinking water slows the progression of the disease with a decrease in TNF-α. There are a limited number of randomized controlled clinical trials conducted investigating polyphenols' anti-inflammatory effects with the results being inconsistent.

In a clinical trial studying the efficacy of curcumin-phosphatidylcholine complex in OA patients it was found that after 3 months of administration, IL-1β and IL-6 decreased significantly in the treatment group (Belcaro G et al., 2010). A cross-sectional

study of overweight individuals with OA showed that individuals who consumed botanical citrus extracts (*P. amurense* bark and *C. sinensis* peel) for 8 weeks have decreased body weight and blood pressure, decreased joint pain, and significantly lower CRP levels compared to a placebo group (Oben et al., 2008). Basu and colleagues administered freeze-dried blueberry powder (50 g per day = 350 g of fresh fruit) to 48 individuals with metabolic syndrome for 8 weeks. At the end of the intervention, they found no significant change in fasting plasma high sensitivity hs-CRP, IL-6, and adiponectin in the intervention group compared to the control group (Basu et al, 2010). Stull et al. investigated blueberries' anti-inflammatory effects in obese men and women with insulin resistance. Freeze-dried blueberry powder was provided to participants, in the quantity of forty five gram per day, in an intervention group for 6 weeks with the powder blended into a yogurt and milk beverage. At the end of the study, there were no differences in changes in circulating hs-CRP or TNF-α in the intervention group compared to the control (Stull et al., 2010). Changes in TNF-α, IL-1β, and IL-6 are not exclusively impacted by local inflammation, but also systemic inflammation, or inflammation in other areas of the body. TNF-α, IL-1β, and IL-6 are proteins that have a very short half-life and as result may not be as accurate reflection of one's average inflammatory status changes post consumption of treatments over 2 or 4 months. If one consumes a highly inflammatory diet the day before a lab visit, one may impact the concentration of these biomarkers. Therefore, other biomarkers of inflammation may need to be identified that can measure average inflammation status over a longer period of time.

Dietary controls may need to be implemented to avoid confounding variables that can also impact the inflammatory biomarkers. Dietary recalls may need to be obtained to ensure compliance, or lack thereof. Dietary controls with intake information required to determine whether dietary intake is a confounding variable that correlates with inflammation biomarkers. Alternatively, more targeted inflammation biomarkers of OA need to be identified in order to capture the changes of inflammation due to OA progression.

Plasma concentrations of IL-10 and IL-13 as well as MMP-3 and MMP-13 did not change significantly during the study period, in either treatment group. IL-10 and IL-13 are anti-inflammatory cytokines. Very few studies have investigated polyphenols' anti-inflammatory effects while observing IL-10 and IL-13 changes in human studies. Most studies focus on *in vitro* studies and animal studies. One *in vitro* study found that Epicatechin gallate, epigallocatechin and epigallocatechin gallate, the major tea polyphenols, decrease the production of IL-1β and enhance the production of IL-10 in human leukocytes (Crouvezier, Powell, Keir, & Yaqoob, 2001). One parallel-designed, placebo-controlled clinical trial reported that intervention with an anthocyanin extract from blueberries (300 mg/d for 3 weeks) significantly reduced the plasma concentration of IL-4, IL-13, IL-8 and IFN-a in a group of 120 healthy men and women aged 40–74 years (Karlsen et al., 2007). MMP-13 and MMP-3 have been recognized as the most important MMPs in OA cartilage destruction. Most studies investigating polyphenols' chondroprotective effects are in vitro or in animals. In an in vitro study, Ahmed *et al*.

showed that pomegranate extracts inhibited IL-1b-induced expression of MMP-1, MMP-3, and MMP-13 in human osteoarthritis chondrocytes (Ahmed et al., 2005).

In a mouse post-traumatic OA model, Leong et al. (2014) found that articular cartilage in the EGCG-treated mice exhibited reduced levels of MMP-1, -3, -8, -13, ADAMTS5, IL-1β, and TNF-α mRNA. In one clinical trial, 38 patients with knee OA were randomly divided into two groups: pomegranate juice (PJ) or control for 6 weeks to evaluate the effect of intervention on clinical signs, inflammation and antioxidant status. Significant decreases in WOMAC total score, stiffness score and physical function score were observed in the PJ group after the intervention. The mean of serum levels of MMP-13 was significantly decreased and glutathione peroxidase was increased in the intervention group compared with the control group after the study period (Ghoochani, Karandish, Mowla, Haghighizadeh, & Jalali, 2016). However, no significant changes in IL-10, IL-13, MMP-3, and MMP-13 were observed in the present study, which is inconsistent with most results from *in vitro*, animal studies and the limited number of human trials. Factors other than treatment alone that can impact plasma concentrations of IL-10, IL-13, MMP-3, and MMP-13, include dietary pattern, physical activity, other issues than articular cartilage break down, or other inflammation issues. This multifactorial impact could potentially explain why no changes were observed in the study.

Weight, height, BMI, and blood pressure were measured at baseline, midpoint, and final point for all participants. In the blueberry group, weight, height, and BMI stayed the same throughout the study, while, in the placebo group, weight, and BMI increased

significantly at final point over midpoint and baseline. The weight increase in the placebo group may be attributable to the additional calories from the placebo powder. Two packets of placebo powder or blueberry powder contains 130 kcal, which is asked to be consumed daily by participates throughout the study. The study period was 120 days. Assuming no significant dietary changes beside the additional 130 kcal/day from the treatment or placebo powder, for all participants over the study period the total additional kcal consumed is approximately 15,600 kcal over the study period. Every additional 3,500 kcal consumed may be translated into 1 pound of weight gain. Therefore, total weight gain per person during the study period could be approximately 4.4 pounds, or about 2 kg. This number is comparable to the total average weight gain of 2.31 kg in the placebo group. Individuals who received blueberry powder consumed the same number of calories from the treatment powder but maintained their weight.

There are two possible reasons to explain this phenomenon from the perspective of energy in and energy metabolism. First, dietary polyphenols may affect neuroregulatory factors that control satiety therefore mediating food intake and energy regulation (Panickar, 2013). In an animal study, investigators tested blueberry extracts and their ability to modify appetite in a rat model and observed reduced food intake over a 4-hour period and weight reduction in the treatment group as compared to the placebo group, after gavaging blueberry extracts 1 ml daily for 6 days. Investigators theorized that the reduced intake is a consequence of a satiating effect, rather than a stomach distension effect (Molan, Lila, & Mawson, 2008). Second, besides a potential effect on regulating satiety, dietary polyphenols may also help regulate body weight by modulating lipid

metabolism and/or increasing basal metabolic rate and thermogenesis (Rupasinghe, Sekhon-Loodu, Mantso, & Panayiotidis, 2016).

Wu et al. (2013) found that dietary supplementation with purified mulberry anthocyanins suppresses body weight gain in a high fat diet fat mouse model. A study has suggested that treatment with anthocyanins increased mitochondrial fatty acid oxidation via the allosteric regulation of carnitine palmitoyl transferase-1, which catalyzes the entry of long-chain fatty acyl-CoA into mitochondria (Hurley et al., 2005). In addition to energy metabolism and satiety regulation, energy expenditure should also be considered when discussing weight changes. There is a potential improvement in functionality, which has been observed in the blueberry group based on the improvement of normal walking paced gait. Better functionality could have a positive effect on exercise level, therefore improved exercise duration or intensity could be another contributor to increased energy expenditure, and therefore better weight management.

Systolic and diastolic blood pressure significantly reduced in the blueberry group at the final point over baseline, while there were no significant changes in the placebo group. The average systolic blood pressure of individuals in the blueberry group was significantly higher than in the placebo group. Participants were randomly assigned into two treatment groups, with the difference in systolic blood pressure at baseline differing by chance. Numerous observational studies have reported on the impact of fruit and vegetable consumption and their positive effect on cardiovascular diseases. Fruits and vegetables are good sources of dietary polyphenols. Elevated blood pressure increases the risk of cardiovascular diseases. Many studies have investigated polyphenol intake and its

effect on blood pressure. In the Nurses' Health Study, intake of fruits and vegetables was inversely associated with systolic and diastolic blood pressure (Ascherio et al., 1996). Studies have also shown that compared with other types of foods, the intake of flavonoid-rich juice significantly reduces blood pressure (Reshef et al., 2005).

In a double blind, placebo controlled parallel trial, Naruszewicz, Laniewska, Millo, & Dłuzniewski (2007) analyzed 44 patients who had survived myocardial infarction and received statin therapy for at least 6 months. The participants were randomized to receive either 3 times 85 mg/day of chokeberry flavonoid extract or a placebo for a period of 6 weeks. At the end of the study, compared to the placebo, individuals in the flavonoid group had significantly reduced systolic and diastolic blood pressure by an average of 11 and 7.2 mmHg, respectively. A cross section study performed within the PREDIMED study correlated total polyphenol intake with blood pressure levels and with the prevalence of hypertension in elderly individuals at high cardiovascular diseases risks. The study found a favorable effect of high total polyphenol intake on blood pressure levels (Zazpe et al., 2008). The results of the blueberry study are consistent with other studies investigating polyphenols' effect on blood pressure.

There are limitations to this study. The drop out rate was high for the study, making significant changes harder to detect. Larger sample sizes may be needed in order to meet the required power of the study. Considering that a high attrition rate is common in human studies, more participants will need to be recruited initially to mitigate a higher attrition rate (more than 18%). Alternatively, intent-to-treat analysis may be used to preserve the sample size, but the analysis needs to be used with caution. This study was

based on participant compliance with consumption of the blueberry or placebo powder. Calendars were provided to participants to help improve compliance but the compliance was self-reported. Follow up calls were made in between study visits to encourage compliance. Participants were asked to mix the blueberry powder or placebo powder with water and consume right after mixing. A few participants reported that the palatability of the powder was less tolerable over time, so they mixed the powder with other drinks such milk or added the powder to their breakfast cereal, which could impact the bioavailability and absorption of polyphenols.

To improve compliance of consumption, and to avoid incorrect mixing methods, pre-mixed bottled blueberry or placebo drink may be made and provided to participants. Empty bottles should be collected at lab visits to check compliance. Diet variations can potentially have an impact on the study outcomes. During the study period, participants were asked to not consume blueberry but did not have other requirements for their diet. To avoid diet variations, participants may be put on a controlled diet during the study period with dietary intake monitored daily or weekly to ensure compliance. Alternatively, a crossover study design can be used to ensure relatively consistent dietary intake between treatment and placebo groups. Dietary questionnaires should be taken at screening to avoid certain dietary patterns in the study, which may potentially affect study outcomes. In the study, no significant differences were observed in the inflammatory and anti-inflammatory biomarkers in the blueberry group. Even though selected biomarkers in the study have been used in many other studies investigating inflammatory changes in OA, most correlations were observed for in vitro and animal

studies, which are well-controlled environments. The human body is a much more complex system with a multiplicity of influences contributing to the variability in treatment outcomes. Therefore, more targeted inflammation biomarkers of knee OA need to be identified and applied in clinical trials. OA biomarker research is active and thriving, which indicates the there is a need to identify more accurate biomarkers that can better capture inflammatory changes in humans (Mobasheri, Bay-Jensen, van Spil, Larkin, & Levesque, 2017).

In summary, the present blueberry research is a distinctive investigation using whole blueberry as a treatment to reduce pain, improve gait, and decrease inflammation in individuals who are suffering from symptomatic knee OA. The findings suggest that blueberries may have positive effects on pain management and improving gait performance, contributing to better physical functionality for OA patients. Future study designs should include a larger sample size, better treatment compliance protocols, and better dietary control. An alternative to a crossover study design may also be used. Also, more targeted inflammation biomarkers may be selected to better capture inflammation changes caused by OA progression.

Table 1.1

Participant Screening and Drop Out Rate

Participants Screened	Qualified and Initiated Treatment	Completed Treatment	Participant Drop Out	Drop Out Rate
103	**63**	**49**	**14**	**22%**

Table 1.2
Demographics of Study Participants

		Baseline	**Midpoint**	**Final**
Treatment A	Male (n)	9	9	9
	Female (n)	24	19	18
	Total (n)	33	28	27
	Avg age	57.67	57.54	56.44
Treatment B	Male (n)	7	5	5
	Female (n)	23	18	17
	Total (n)	30	23	22
	Avg age	55.30	54.52	54.59
Total	Male (n)	16	14	14
	Female (n)	47	37	35
	Total (n)	63	51	49
	Avg age	56.54	56.18	55.61
Drop Rate (%)		NA	19%	22%

Table 1.3
Step and Stride Length Changes Over Time for Both Treatment Groups

	Blueberry			Placebo		
Parameters	Baseline	Midpoint	Final	Baseline	Midpoint	Final
Left step length normal (cm)	55.13±1.58	57.96±1.47*	60.02±1.49*,#	58.06±1.75	60.09±1.62*	60.77±1.65*
Left step length fast (cm)	67.46±2.03	67.17±1.88	66.86±1.82	69.12±2.25	68.15±2.08	68.66±2.02
Right step length normal (cm)	54.91±1.60	58.06±1.49*	60.38±1.62*,#	58.05±1.77	60.76±1.65*	60.69±1.79*
Right step length fast (cm)	67.20±2.06	67.28±1.83	66.50±1.89	70.03±2.28	69.17±2.03	69.63±2.09
Left stride length normal (cm)	110.22±3.11	116.27±2.90*	120.69±3.04*,#	116.39±3.45	121.00±3.22*	121.67±3.36*
Left stride length fast (cm)	134.88±3.99	134.66±3.61	133.57±3.63	139.46±4.42	137.38±4.00	138.34±4.03
Right stride length normal (cm)	110.22±3.15	116.37±2.91*	120.60±3.06*,#	116.29±3.49	121.12±3.23*	121.64±3.39*,#
Right stride length fast (cm)	134.80±4.04	134.76±3.66	133.66±3.66	139.53±4.47	137.81±4.05	138.91±4.05

Note: N=27 for the blueberry group, N=22 for the placebo group
*, significance as compared to baseline ($p<0.05$); #, significance as compared to midpoint ($p<0.05$)

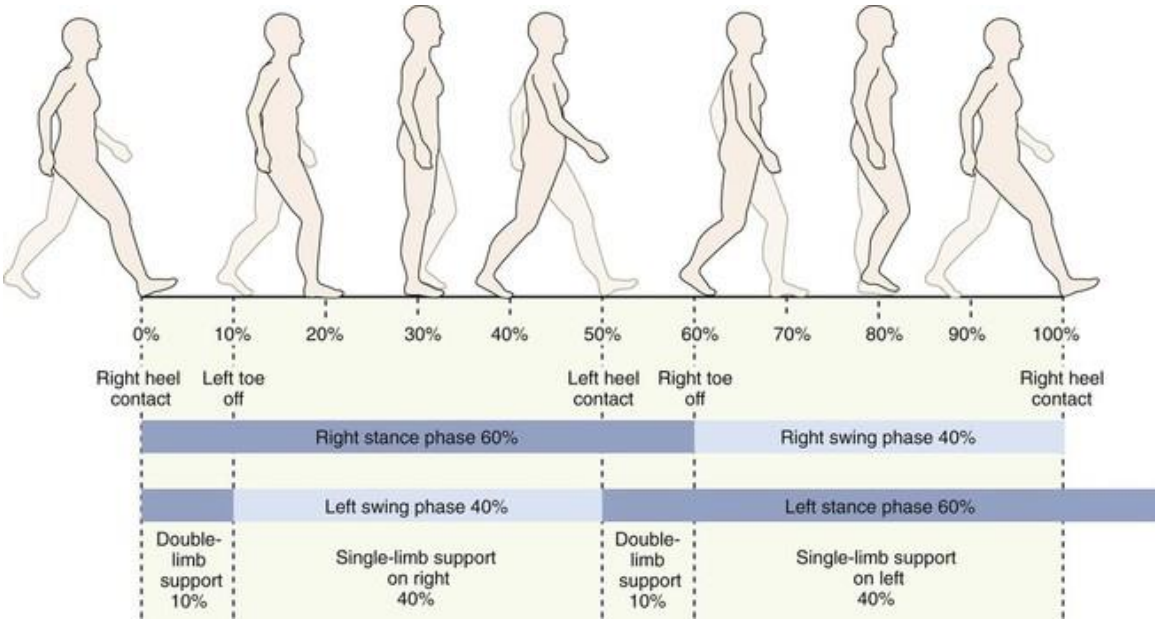


Fig 1.0. Normal Walking Gait Cycle

Picture from Clinical Gate

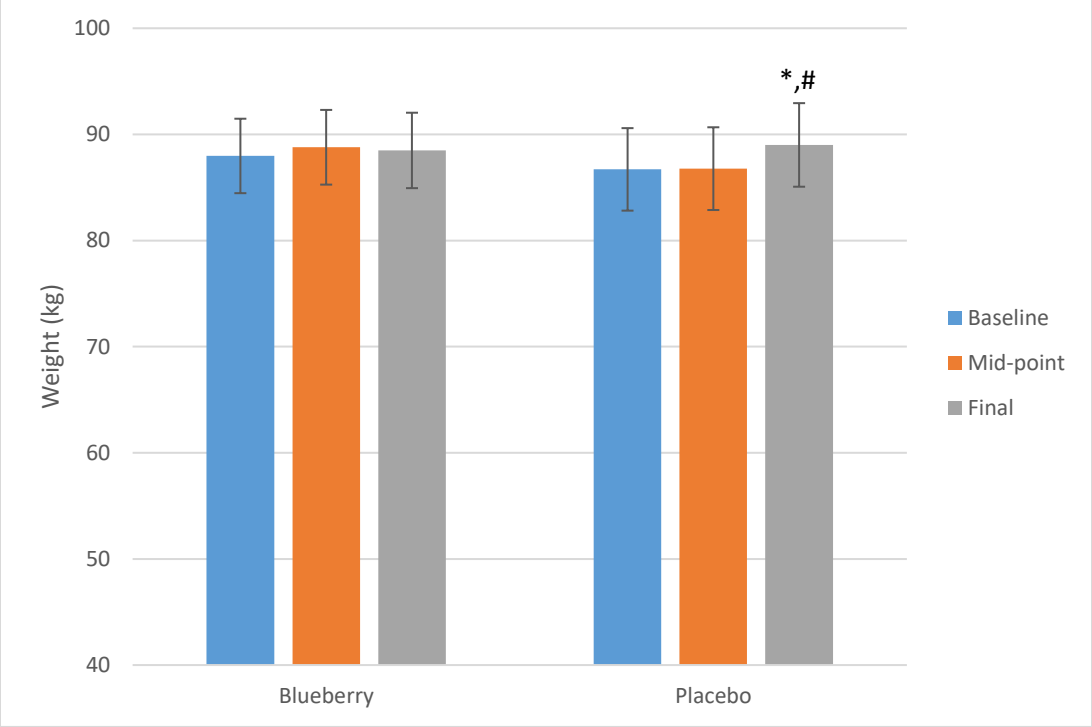


Figure 1.1 Weight. Mean ± SEM. N=27 for the blueberry group, N=22 for the placebo group.
*, significance as compared to baseline ($p<0.05$); #, significance as compared to midpoint ($p<0.05$).
The mean difference between final point and baseline is a net change of +2.31 kg.

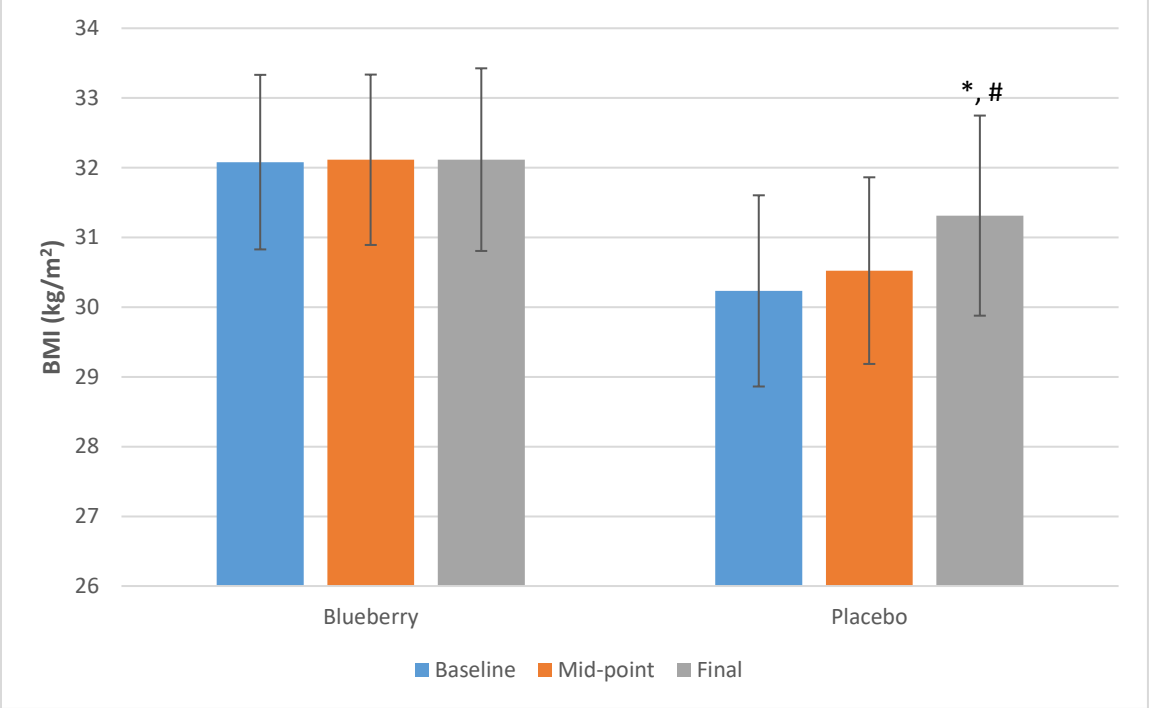


Figure 1.2 BMI. Mean ± SEM. N=24 for the blueberry group, N=20 for the placebo group. Outliers were omitted.
*, significance as compared to baseline ($p<0.05$); #, significance as compared to midpoint ($p<0.05$).
The mean difference between final point and baseline is a net change of +1.10kg/m^2.

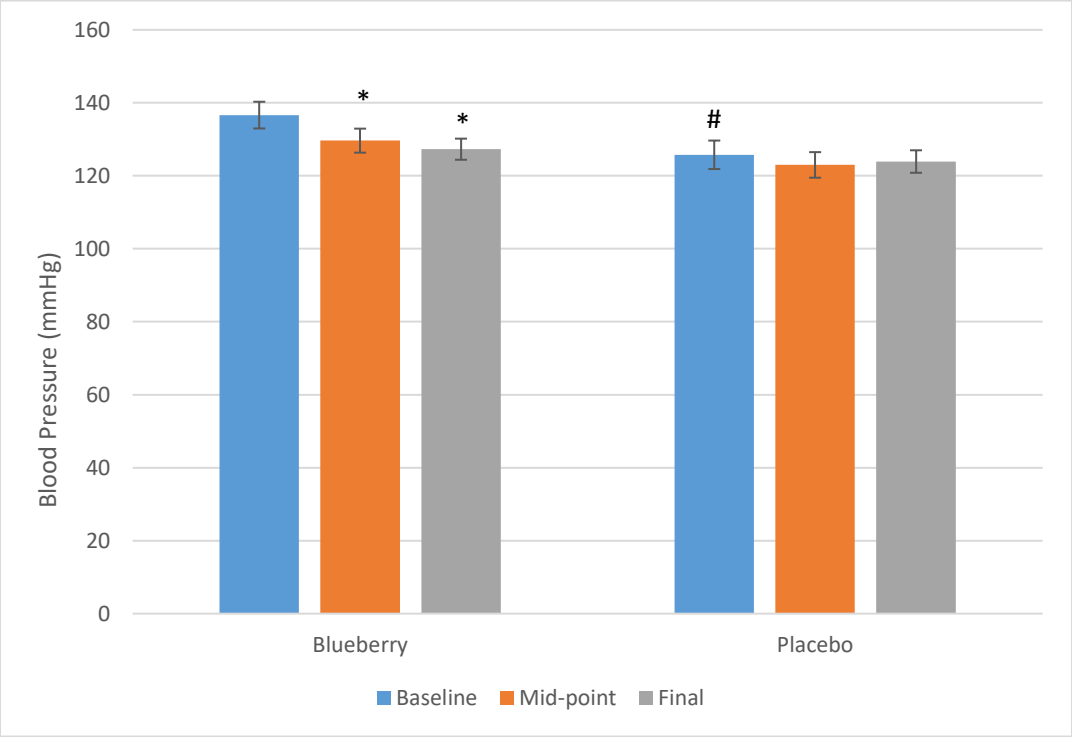


Figure 1.3 Systolic Blood Pressure. Mean ± SEM. N=25 for the blueberry group, N=22 for the placebo group. Outliers were omitted.
*, significance as compared to baseline ($p<0.05$); #, Placebo Baseline vs. Blueberry group is significantly different ($p<0.05$)

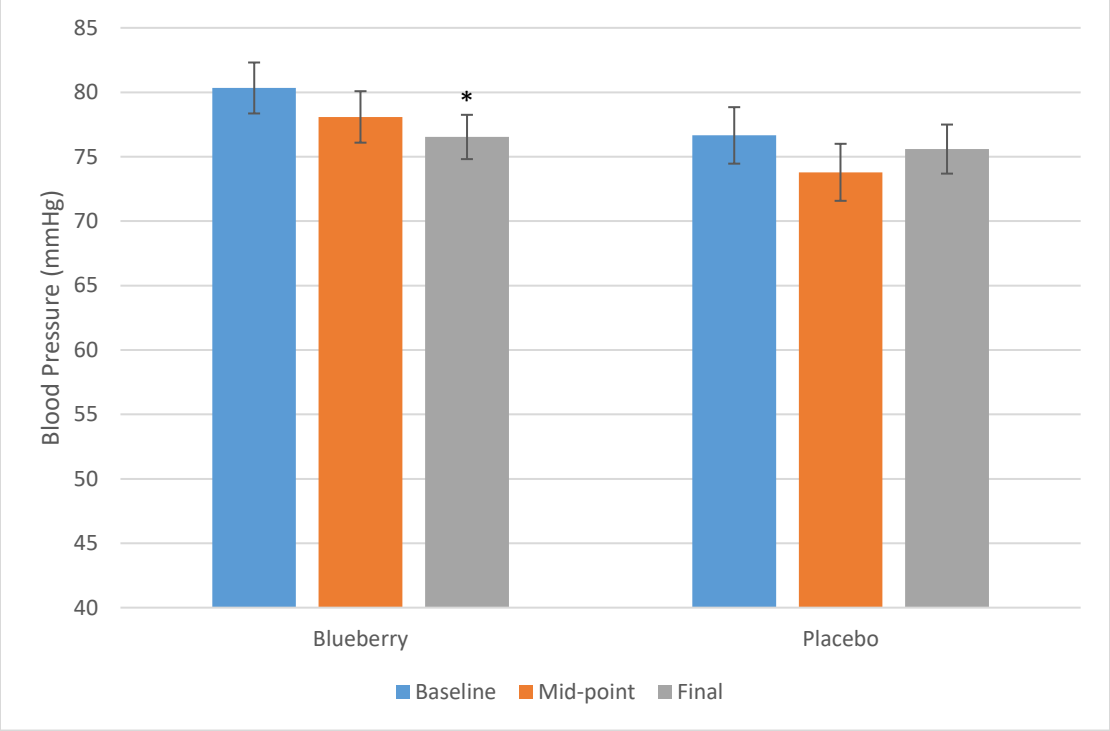


Figure 1.4 Diastolic Blood Pressure. Mean ± SEM. N=27 for the blueberry group, N=22 for the placebo group. Outliers were omitted.
*, significance as compared to baseline ($p<0.05$).

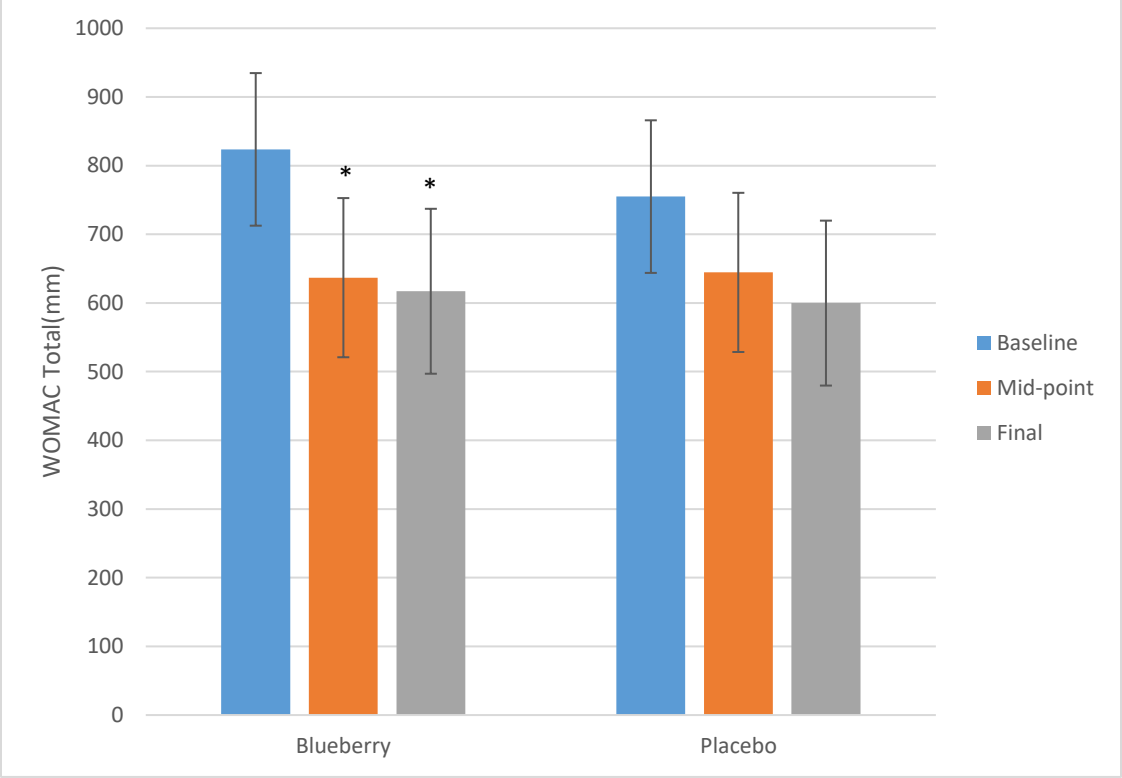


Figure 1.5WOMAC total. Mean ± SEM. N=22 for the blueberry group, N=22 for the placebo group.
*, significance as compared to baseline ($p<0.05$).

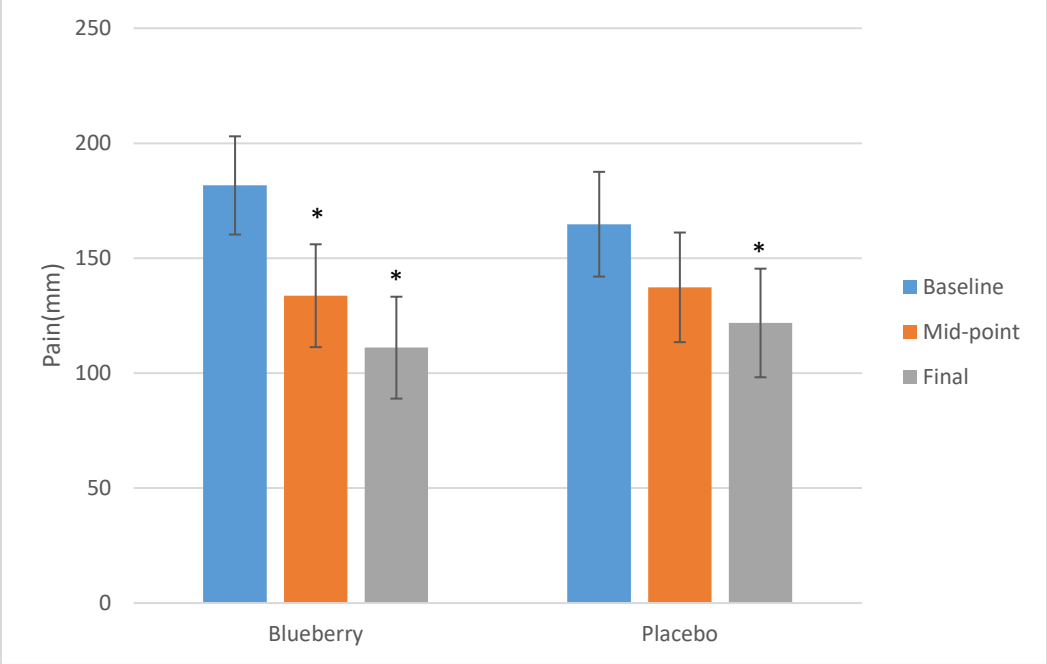


Figure 1.6 WOMAC sub-group Pain. Mean ± SEM. N=25 for the blueberry group, N=22 for the placebo group
*, significance as compared to baseline ($p<0.05$).

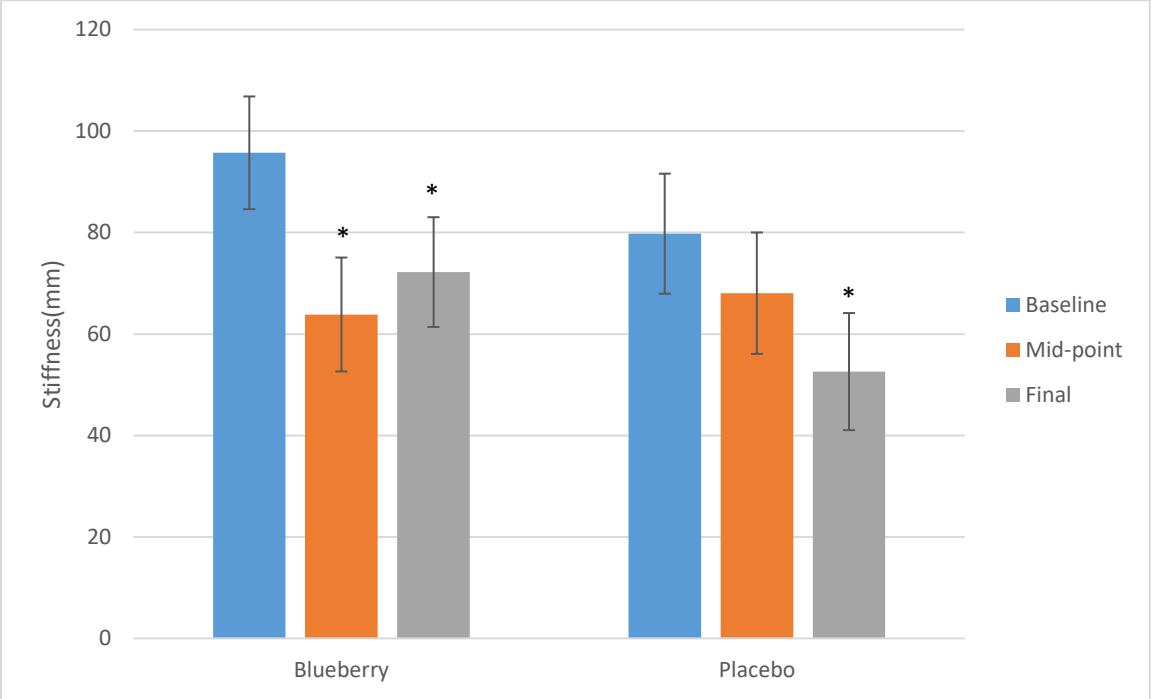


Figure 1.7 WOMAC sub-group Stiffness. Mean ± SEM. N=25 for the blueberry group, N=22 for the placebo group
*, significance as compared to baseline ($p<0.05$).

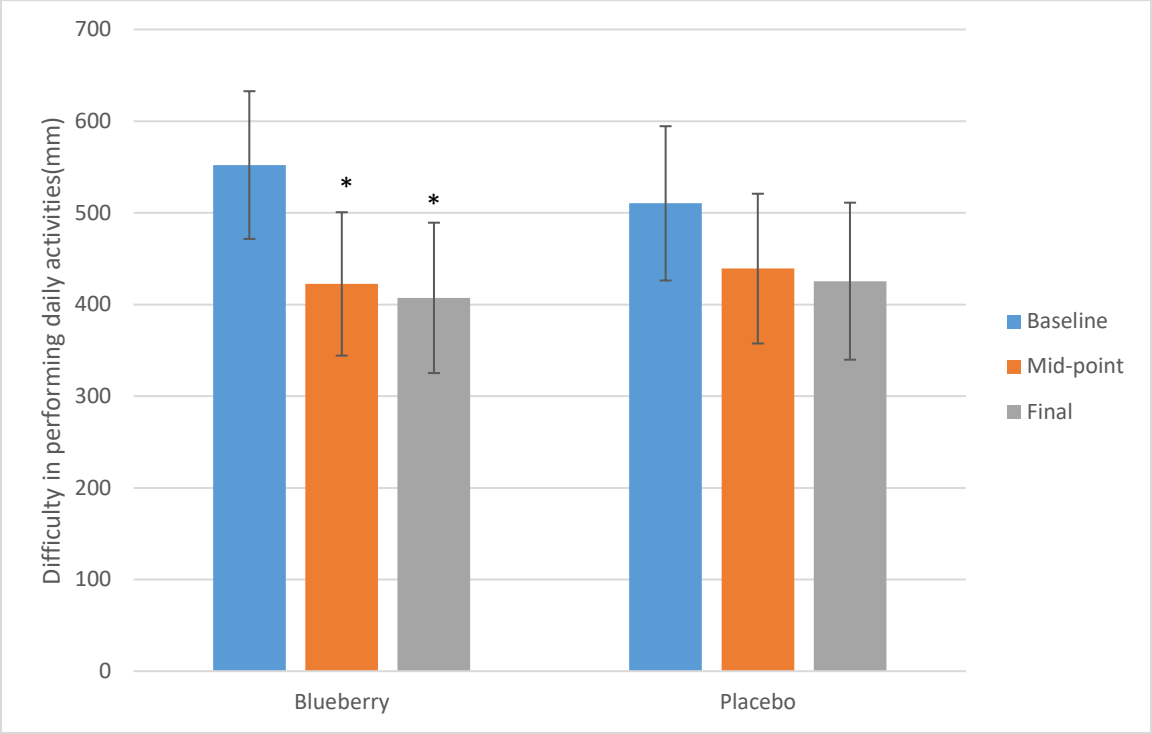


Figure 1.8 WOMAC sub-group Difficulty to Perform Daily Activity. Mean ± SEM. N=24 for the blueberry group, N=22 for the placebo group
*, significance as compared to baseline ($p<0.05$).

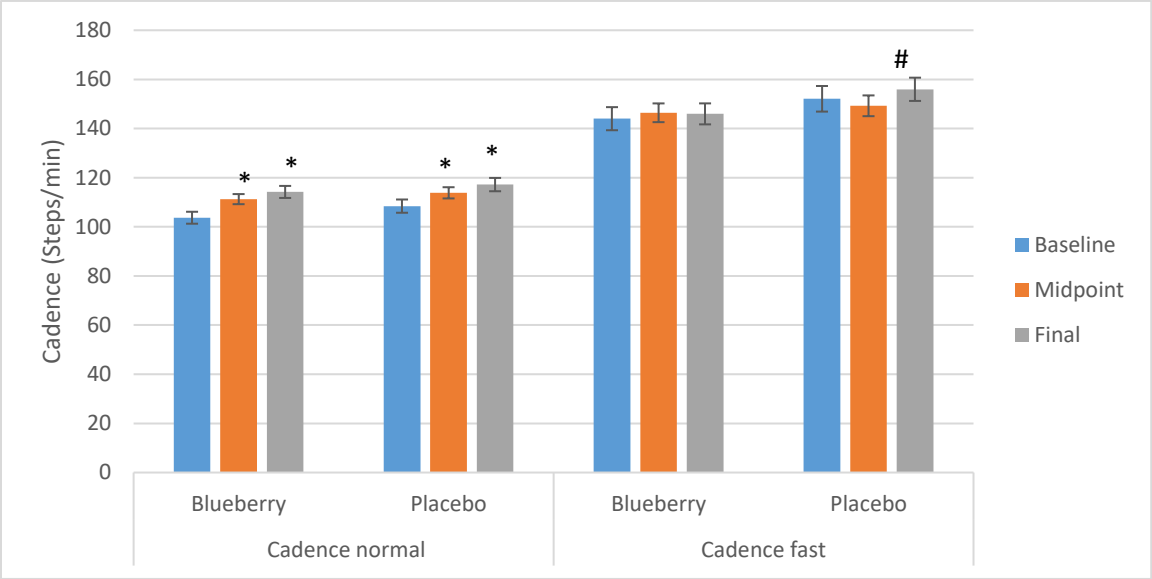


Figure 1.9 Cadence. Mean ± SEM. N=27 for the blueberry group, N=22 for the placebo group
*, significance as compared to baseline ($p<0.05$); #, significance as compared to midpoint ($p<0.05$).

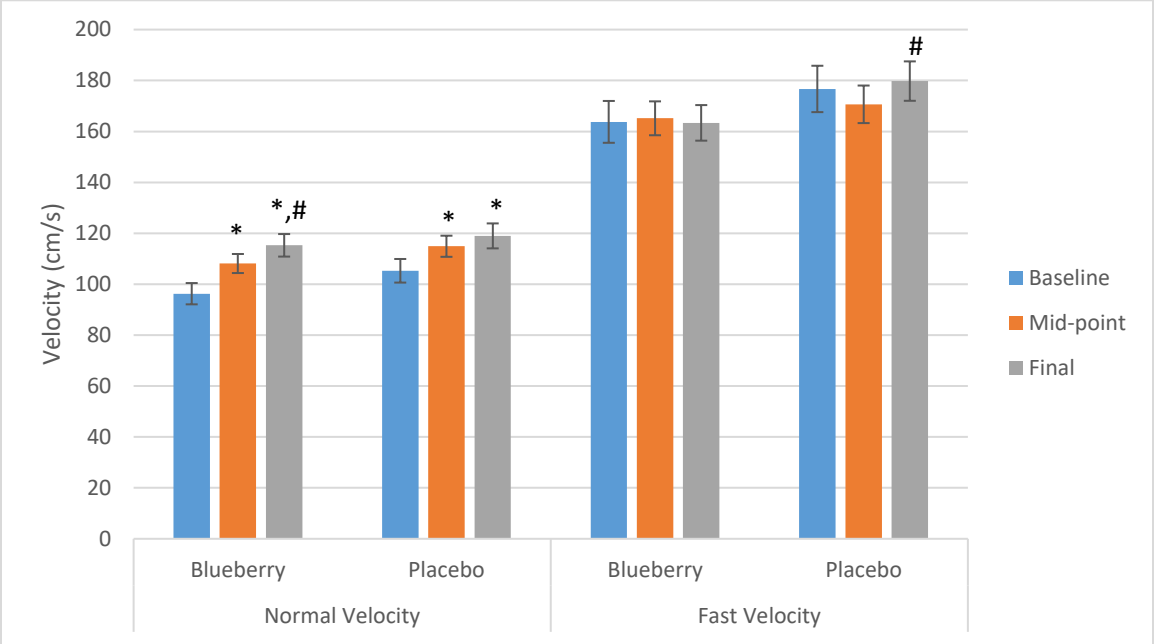


Figure 1.10 Velocity. Mean ± SEM. N=27 for the blueberry group, N=22 for the placebo group
*, significance as compared to baseline ($p<0.05$); #, significance as compared to midpoint ($p<0.05$).

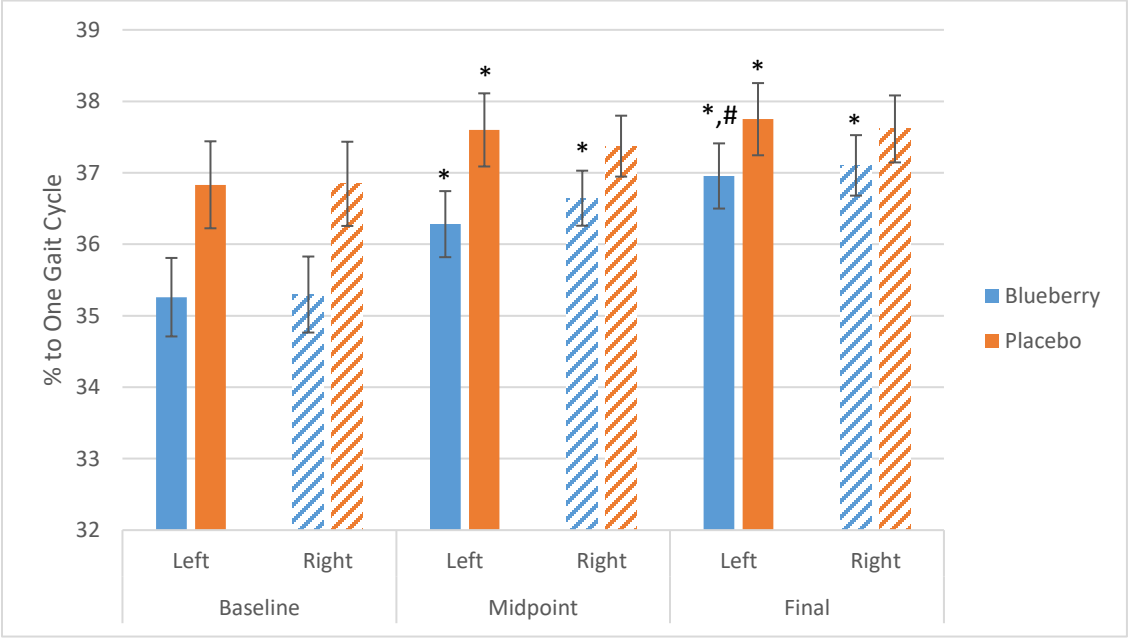


Figure 1.11 Normal paced walking single support percentage to one gait cycle. Mean ± SEM. N=27 for the blueberry group, N=22 for the placebo group
*, significance as compared to baseline ($p<0.05$); #, significance as compared to midpoint ($p<0.05$)

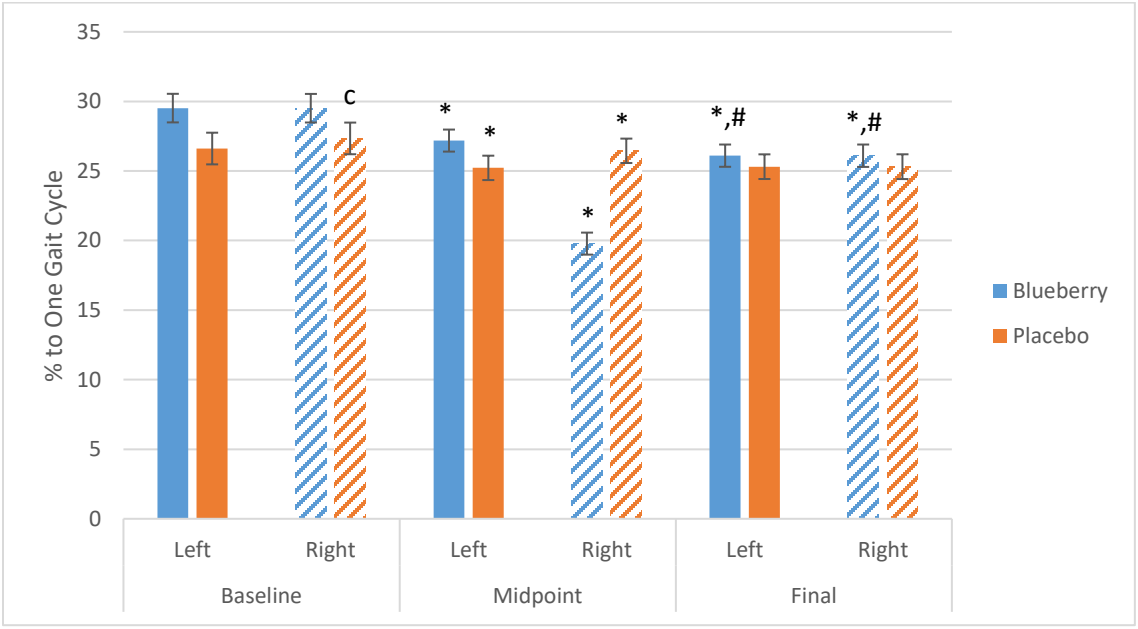


Figure 1.12 Normal paced walking double support percentage to one gait cycle. Mean ± SEM. N=27 for the blueberry group, N=22 for the placebo group
* Reached significance from baseline to midpoint ($p<0.05$)
*, significance as compared to baseline ($p<0.05$); #, significance as compared to midpoint ($p<0.05$)
c, Baseline Right Placebo vs. Blueberry group is significantly different ($p<0.05$)

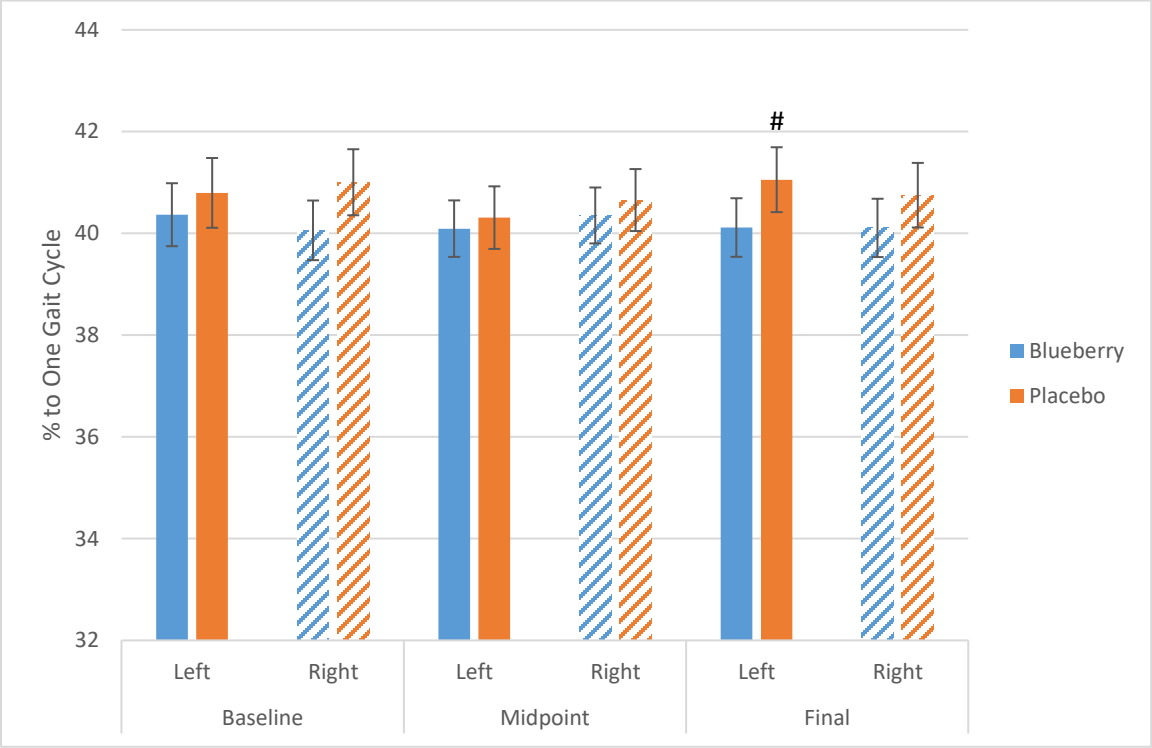


Figure 1.13 Fast-paced walking single support percentage to one gait cycle. Mean ± SEM. N=27 for the blueberry group, N=22 for the placebo group
#, significance as compared to midpoint ($p<0.05$)

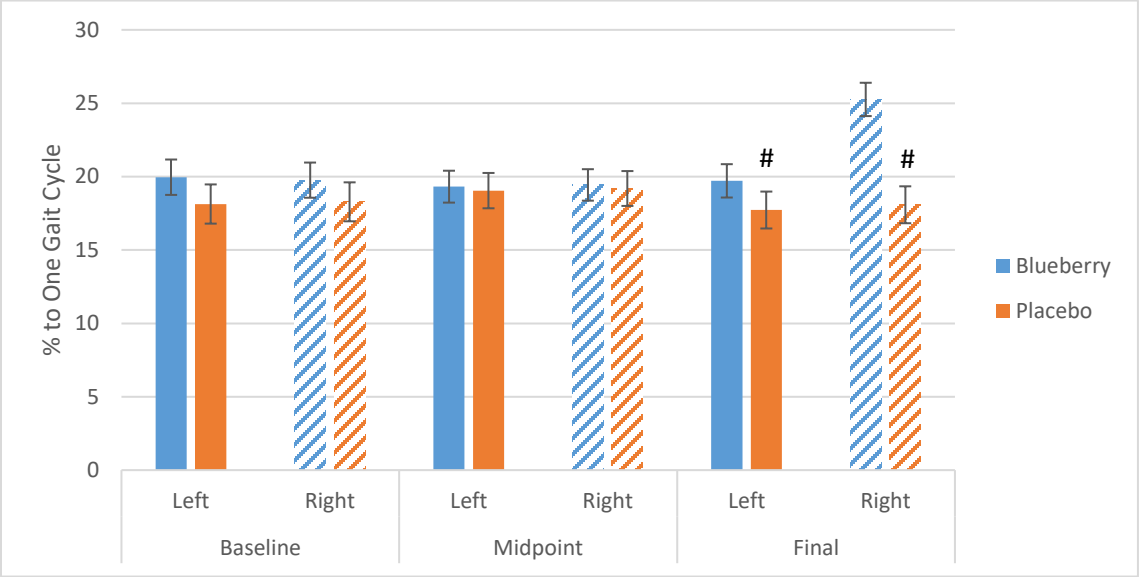
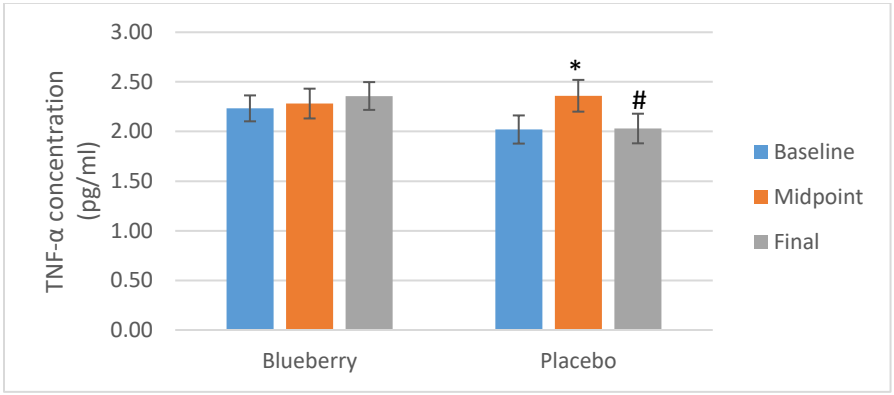
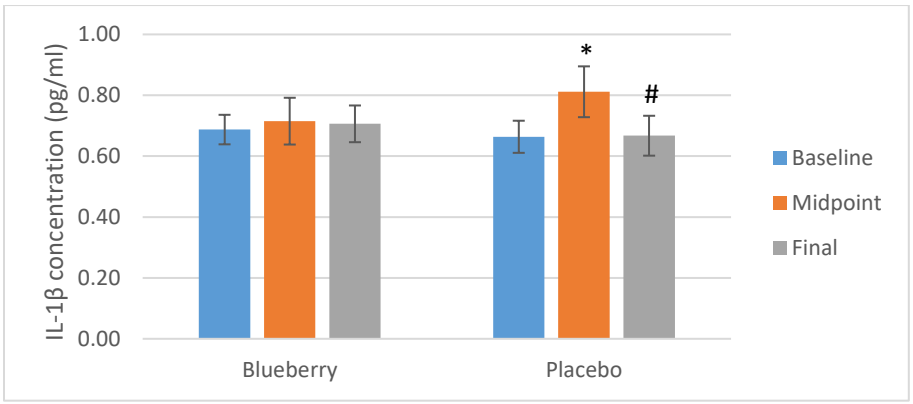


Figure 1.14 Fast-paced walking double support percentage to one gait cycle. Mean ± SEM. N=27 for the blueberry group, N=22 for the placebo group
#, significance as compared to midpoint ($p<0.05$)





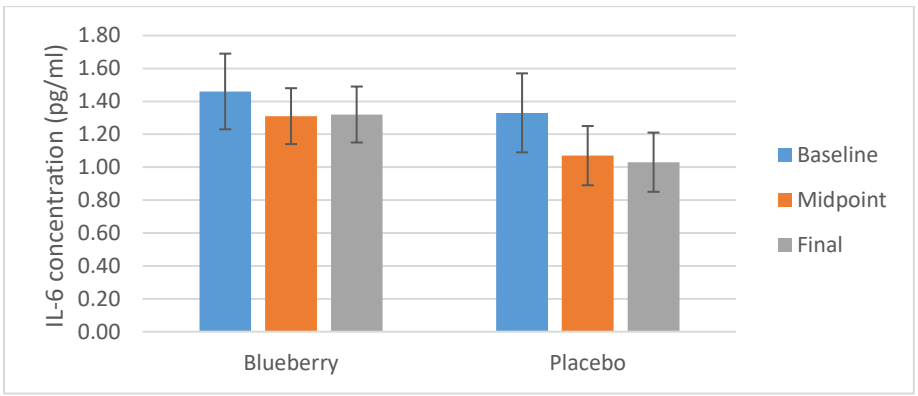


Figure 1.15 TNF-α, IL-1β, and IL-6. Mean ± SEM. TNF-α, N= 26 for the blueberry group, N=21 for the placebo group; IL-1β, N= 26 for the blueberry group, N=22 for the placebo group; IL-6, N= 22 for the blueberry group, N=20 for the placebo group
*, significance as compared to baseline ($p<0.05$); #, significance as compared to midpoint ($p<0.05$).

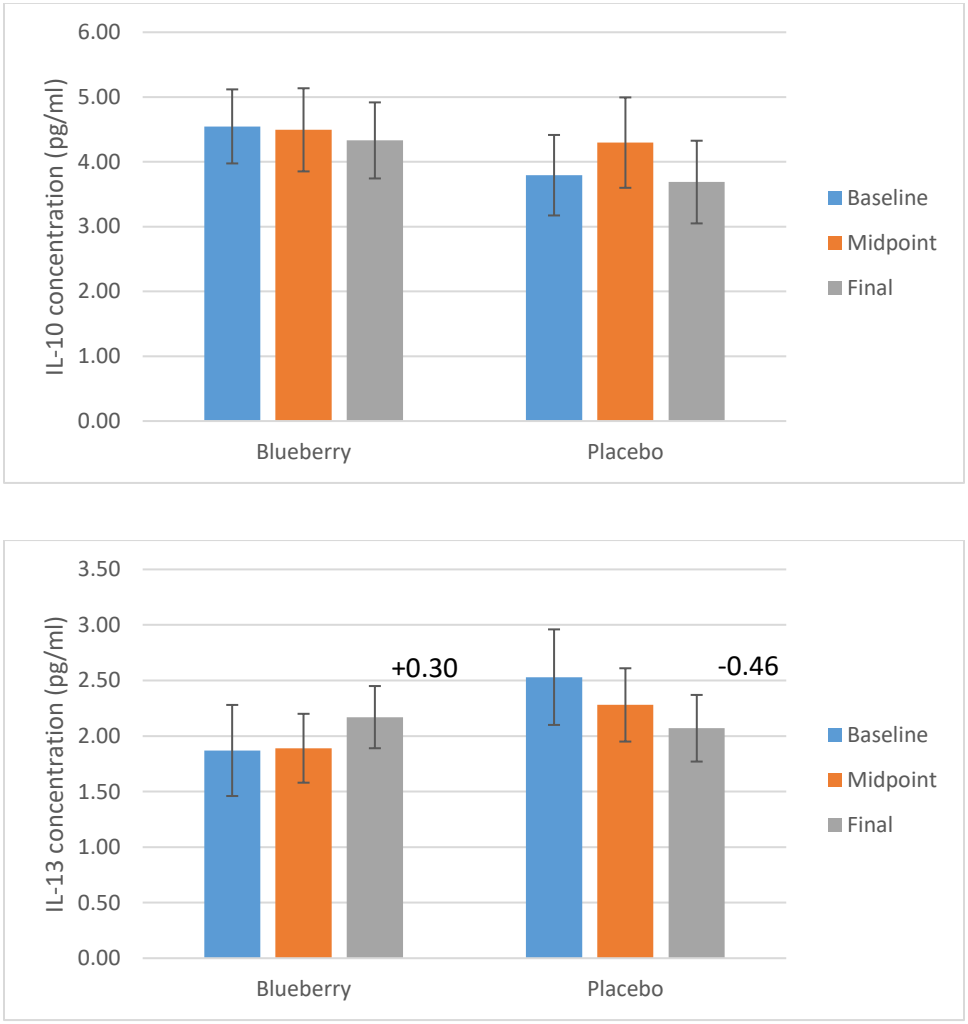


Figure 1.16 IL-10 and IL-13. Mean ± SEM. N=26 for the blueberry group, N=22 for the placebo group. No significant changes over time and between groups at any time point.

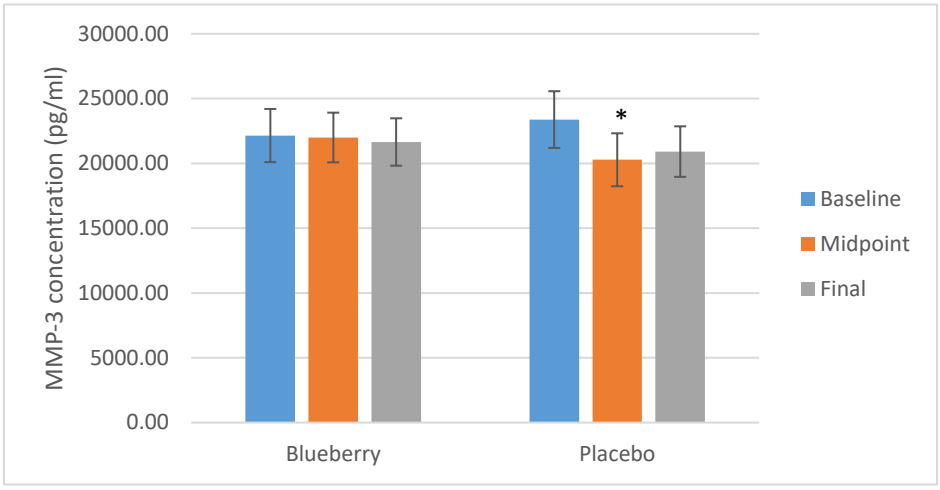


Figure 1.17 MMP-3. Mean ± SEM. N=26 for the blueberry group, N=22 for the placebo group
*, significance as compared to baseline ($p<0.05$)

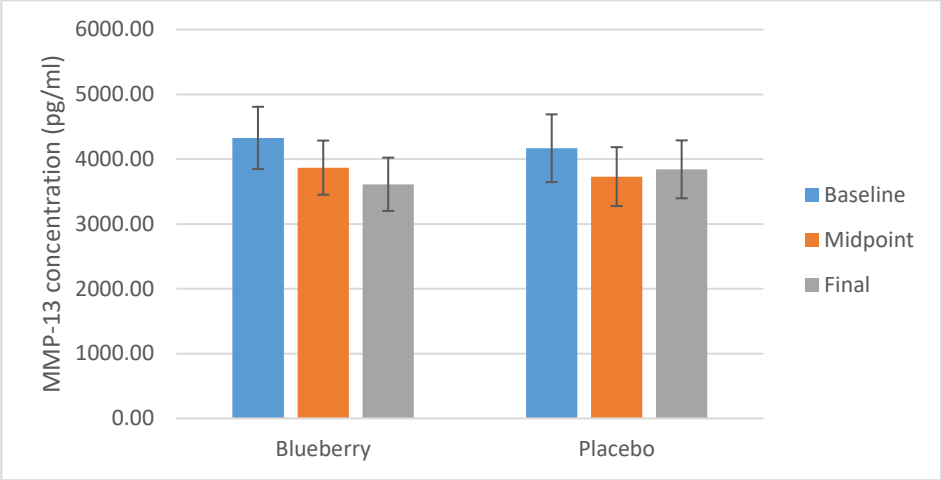


Figure 1.18 MMP-13. Mean ± SEM. N=26 for the blueberry group, N=22 for the placebo group
No significant changes over time and between groups at any time point.

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APPENDIX A

PARTICIPATION RECRUITMENT FLYER

Need Research Volunteers

Do you have KNEE Pain?

- Are you between 45 – 79 years old
- Are you otherwise healthy and mobile
- Would you be willing to participate in a study where you may be asked to consume blueberry powder daily for 4 months

If you have answered **YES** to all of the above, then you may be eligible to participate in a 4 month research study to look at the beneficial effect of blueberry in improving joint function and reducing pain associated with knee osteoarthritis.

Criteria include meeting the requirements listed above and willing to consume either whole blueberry powder or a powder without blueberry for a period of 4 months. There will be blood draws at the start, midpoint (2 months) and at the end (4 months) of the study. Pain and joint flexibility will be assessed at start of study, midpoint, and end of study using questionnaires and range of motion measurement. The total time you need to spend for the study is 3 hours over 4 months involving 3 visits.

Benefits include: promotion of knee joint health, measurements of height and weight, pain and stiffness assessment and range of motion. Blood and urine markers of cartilage health will be evaluated. Upon completion, you will receive a compensation of $100 for your time in partial payments of $50 at midpoint (2 months) and final follow-up visits.

If interested, please email or call for more information:
Dr. Shanil Juma, Department of Nutrition and Food Sciences
sjuma@twu.edu; 940-898-2704

There is a potential risk of loss of confidentiality in all email, downloading, and internet transactions.

APPENDIX B

SCREENING QUESTIONAIRE

Screening Questionnaire

ID:	Sex:	Age:
Telephone(s):	e-mail:	
Do you smoke?: _____ Yes _____ No	Cigarettes per day _____	
Medical condition you are taking medicine for:		
Hypertension ___ High cholesterol ___ Kidney disease ____ Lung disease ____ Diabetes ___ Heart disease ____ Liver disease ___ Thyroid condition ____ Bone Condition _____		
List any medications, drugs, prescription drugs, over the counter drugs, vitamins or food Supplements you are taking: List amount (mg) and times taken (daily, weekly etc.)		
Are you on a special diet? __No __weight loss __Medical condition __ Vegetarian		
___ Low salt ____ Low cholesterol ____ Weight gain		
Do you have any food allergies? ___ No ___Yes (list them)		
Here is the list of items (drugs/foods) you, as the participant, will be exposed to during the study: Whole freeze-dried blueberry powder or Placebo powder without blueberry		

APPENDIX C

INFORMED CONSENT AND IRB APPROVAL

Texas Woman's University
Consent to Participate in Research

Study Title: Beneficial Effect of Whole Blueberry Consumption on Joint Flexibility, Mobility, and Pain Symptoms Associated with Knee Osteoarthritis

Investigators: Shanil Juma, PhD 940-898-2704 (sjuma@twu.edu)
Young-Hoo Kwon, PhD 940-898-2598 (ykwon@twu.edu)
Parakat Vijayagopal, PhD 940-898-2709 (pvijayagopal@twu.edu)

Explanation and Purpose of Research

We are asking you to participate in a research study at Texas Woman's University. The purpose of the study is to find out if consumption of freeze dried blueberry powder for 4 months will improve pain, stiffness and flexibility associated with self-reported knee osteoarthritis. We will ask the following questions:

a) Will consuming freeze-dried blueberry powder for 4 months improve joint flexibility?
b) Will consuming freeze-dried blueberry powder to reduce pain and stiffness in the knee joint?

Research Procedures

For this study, the baseline visit will first involve obtaining consent for your participation in this study. As part of the consent, you agree that you will not initiate any new therapies associated with the osteoarthritis of the knee during the duration of the treatment period. If you do decide to initiate a new therapy, please contact the principal investigator to determine if you still qualify to continue participating in this study.

During the baseline visit you will be asked to come fasted (not to eat any food overnight or at least 10 hours). A phlebotomist (person taking the blood) will draw 3 table spoons of your blood from one of the veins of your arms. We will then provide you with a snack and drink (cookies, crackers, and orange juice). This will be followed with a spot urine collection. A sterile specimen cup will be provided to collect a small urine specimen after the first morning void. A trained personnel of the same gender will take your height and weight measurements. Filtered water and a light snack will be available for you at the study site. We will also ask you to complete a food frequency and physical activity questionnaire regarding your eating and activity habits over the past week. You will complete a questionnaire regarding pain and stiffness. A measurement of knee motion (flexibility) will be done in a lying down position on a patient table and repeated three times during this visit by a trained personnel of the same gender associated with the study. A gait analysis to evaluate walking parameters will be done by trained personnel with instructions to walk short distance (30 feet) at usual speed and fastest speed. Each walking speed will be repeated three times with a 30 second rest between each walk. At the end of the baseline visit, you will be randomly assigned to a treatment based on chance, like a flip of a coin. Neither you nor the researcher chooses your assigned treatment group. You will have an equal chance of being in either group. You will be

Participant Initials _____

provided a 60 day supply of either the study treatment (freeze dried blueberry powder packaged in pouches) or a control (comparative placebo freeze-dried powder without blueberry). At the 60 day visit (midpoint), you will again be asked not to eat any food overnight (10 hours). A trained female personnel will take your height and weight measurements. A phlebotomist (person taking the blood) will draw 3 table spoons of your blood from one of the veins of your arms. We will then provide you with a snack and drink (cookies, crackers, and orange juice). A spot urine specimen will be obtained in a sterile specimen cup. Filtered water and a light snack will be available for you at the study site. We will also ask you to complete a food frequency and physical activity questionnaire regarding your eating and activity habits over the past week. You will complete a questionnaire regarding pain and stiffness. A measurement of knee motion (flexibility) will be done in a lying down position on a patient table and repeated three times during this visit by a trained personnel of the same gender associated with the study. Similar to baseline, a gait analysis will be done by trained research personnel. You will again be provided with a 60 day supply of either the study treatment (freeze-dried blueberry powder in pouches) or a control (comparative placebo freeze dried powder without blueberry). At the end of the study (6 months), you will be asked to come in for your last visit and not to eat any food overnight (10 hours) for a blood draw (3 tablespoons of blood will be obtained). You will be provided with snacks and filtered water. A spot urine specimen will be obtained. A trained research personnel of the same gender will measure height and weight. We will also ask you to complete a food frequency and physical activity questionnaire regarding your eating and activity habits over the past week. You will complete a questionnaire regarding pain and stiffness. A measurement of knee motion (flexibility) will be done in a lying down position on a patient table and repeated three times during this visit by a trained personnel of the same gender associated with the study. A gait analysis to evaluate walking parameters will be done by trained personnel

Time Commitment

The study period is 4 months. The study volunteer time commitment includes initial screening questionnaire (~10 min), consent form (20 minutes), pain, stiffness, physical activity, and diet questionnaires (~30 minutes each during baseline, 2 months, and final), flexibility assessment (10 minutes each during baseline, 2 months, and final), gait assessment (10 minutes each during baseline, midpoint, and final), anthropometrics-height and weight (5 minutes each during baseline, 2 months, and final), and blood draw and spot urine (10 minutes each at baseline, midpoint and final). Total time commitment for each participant is approximately 3 hours 45 minutes over the three study visits.

Potential Risks

A potential risk to you as a participant in this study is release of confidential information. Confidentiality will be protected to the extent that is allowed by law. To protect confidentiality, you will be given a code number which will be used in all records. Only Dr. Juma will know your identity. All records will be stored in a locked filing cabinet in Dr. Juma's office. The records will be shredded within 5 years of completion of the study. Your name or any other identifying information will not be included in any publication that

may result from the study. There is a potential risk of loss of confidentiality in email, downloading, and internet transactions.

A second possible risk is that you may not like the freeze-dried blueberry powder or the comparative placebo powder without blueberry. If you do not like the randomized treatment, there is no penalty for not consuming it. You are free to quit the study at any time. Freeze dried blueberry powder or the placebo powder without blueberry is from a whole fruit source or equivalent to the sugar content of the whole fruit that has been custom prepared and packaged for our study. It has been previously used in other human clinical studies and is deemed safe for consumption and not harmful in any way.

Another possible risk to you as a participant in this study includes the discomfort of blood drawings. The phlebotomist will ask you about any concerns or previous issues with having a blood draw. If there are serious concerns or reactions to blood draw, we will ask you that you have the option to withdraw from participating in the study at any time. Blood draw may cause minor pain, bruising, discomfort, swelling, anxiety, infection or fainting. We will use a certified expert for blood draw. This will minimize the possibility of pain, bruising, discomfort, swelling, infection, and anxiety. A light snack and water will be made available at the draw site to avoid fainting.

Study volunteers will receive time to relax before and after blood draw. They will be offered the opportunity to watch television to reduce anxiety. If a participant faints during the blood draw, investigators will assist in laying him/her down and making him/her comfortable and providing any medical assistance if necessary. We will carefully watch the person until she regains consciousness and will not make another attempt to draw the person's blood again that day. We will also ask you to drink a lot of water before the blood draw.

You may be allergic to the latex gloves the phlebotomist wears for blood draw. In that case, the phlebotomist will use a different type of gloves. You will receive time to relax before and after blood draw. A light snack and water will be available to you. This will reduce the possibility of your fainting. If you faint during the blood draw, we will lay you down and make you comfortable. We will carefully watch you until you regain consciousness and will not make another attempt to draw your blood again that day.

Other possible risks to you are loss of time, fatigue, allergic reaction, and infection. You can watch videos or relax while you are waiting. Before we select you for the study, we will ask whether you are allergic to the food we use in the study. If you are allergic, we will not select you for the study. The phlebotomist will clean your arm with alcohol before taking blood and she will use a new needle. This will minimize the possibility of infection.

In addition to the risks above, you may experience anxiety or embarrassment related to height, weight, range of motion, and gait assessment. In order to minimize this risk, you will be assured of complete confidentiality before taking these measurements. All measurements will be taken only by experienced and trained personnel of the same gender in a private room. Anthropometrics (height and body weight) measurements will be

conducted by trained personnel of the same gender. Blood draw will be done by a trained and experienced phlebotomist. Flexibility and gait analysis will be done with research personnel of the same gender who will describe the procedure and address any questions that you may have before the assessment is done.

The study treatment consists of whole blueberry that has been freeze-dried into a powder and the comparative placebo powder containing sugar equivalent to the blueberry treatment without blueberries. If participants are allergic to blueberries or sugar found in blueberries he or she may consider not participating in the study. If any participant becomes allergic to either of the treatment powders used in the study, she can withdraw from the study at any time.

The researchers will try to prevent any problem that could happen because of this research. You should let the researchers know at once if there is a problem and they will help you. However, TWU does not provide medical services or financial assistance for injuries that might happen because you are taking part in this research.

Participation Benefits

Your participation in this research study is completely voluntary, and you may discontinue your participation in the study at any time without penalty. As a participant in the study, you will receive the study powder for 4 months. You will also receive a cash incentive of $100.00, of which $50 will be paid at midpoint (60 days) and the remaining $50 after you complete the study. In addition, at completion of the study a summary of results as well as the results of your blood analysis will be mailed to you upon request. *

Questions Regarding the Study

You will be given a copy of this signed and dated consent form to keep. If you have any questions about the research study you may ask the researchers; their phone numbers are at the top of this form. If you have questions about your rights as a participant in this research or the way this study has been conducted, you may contact the Texas Woman's University Office of Research and Sponsored Programs at 940-898-3378 or via e-mail at IRB@twu.edu.

_____ _____

Signature of Participant Date

This page will be detached and filled separately.

* If you would like to receive a summary of the results of this study, please provide an address to which this summary should be sent:

_____
_____
_____

APPENDIX D

PROTOCOL APPROVAL LETTER

Institutional Review Board
Office of Research and Sponsored Programs
P.O. Box 425619, Denton, TX 76204-5619
940-898-3378 email: IRB@twu.edu
http://www.twu.edu/irb.html

DATE: March 4, 2016

TO: Dr. Shanil Juma
Nutrition & Food Sciences

FROM: Institutional Review Board (IRB) - Denton

Re: Approval for Beneficial Effect of Whole Blueberry Consumption on Joint Flexibility, Mobility, and Pain Symptoms Associated with Knee Osteoarthritis (Protocol #: 18955)

The above referenced study was reviewed at a fully convened meeting of the Denton IRB (operating under FWA00000178). The study was approved on 3/4/2016. This approval is valid for one year and expires on 3/4/2017. The IRB will send an email notification 45 days prior to the expiration date with instructions to extend or close the study. It is your responsibility to request an extension for the study if it is not yet complete, to close the protocol file when the study is complete, and to make certain that the study is not conducted beyond the expiration date.

If applicable, agency approval letters must be submitted to the IRB upon receipt prior to any data collection at that agency. A copy of the approved consent form with the IRB approval stamp is enclosed. Please use the consent form with the most recent approval date stamp when obtaining consent from your participants. A copy of the signed consent forms must be submitted with the request to close the study file at the completion of the study.

Any modifications to this study must be submitted for review to the IRB using the Modification Request Form. Additionally, the IRB must be notified immediately of any adverse events or unanticipated problems. All forms are located on the IRB website. If you have any questions, please contact the TWU IRB.

cc. Dr. Shane Broughton, Nutrition & Food Sciences

APPENDIX E

WOMAC QUESTIONAIRE

WOMAC OSTEOARTHRITIS INDEX VERSION VA3.1

INSTRUCTIONS TO PATIENTS

In Sections A, B, and C questions are asked in the following format. Please mark your answers by putting an " ✗ " through the horizontal line.

EXAMPLES:

1. If you put your " ✗ " at the left-hand end of the line as shown below, then you are indicating that you feel **no** pain.

2. If you put your " ✗ " at the right-hand end of the line as shown below, then you are indicating that you feel **extreme** pain.

3. Please note:
   a) that the further to the right you place your " ✗ ", the **more** pain you feel.
   b) that the further to the left you place your " ✗ ", the **less** pain you feel.
   c) **please do not** place your " ✗ " **past either end of the line**.

You will be asked to indicate on this type of scale the amount of pain, stiffness or disability you have felt during the last 48 hours.

Think about your _____ (study joint) when answering the questions. Indicate the severity of your pain and stiffness and the difficulty you have in doing daily activities that you feel are caused by the arthritis in your _____ (study joint).

Your study joint has been identified for you by your health care professional. If you are unsure which joint is your study joint, please ask before completing the questionnaire.

WOMAC VA3.1 QUESTIONNAIRE **WOMA**

Section A

PAIN

Think about the pain you felt in your _____ (study joint) caused by your arthritis during the last 48 hours.

(Please mark your answers with an " ✗ ".)

QUESTION: **How much pain have you had . . .**	Study Coordinator Use Only
1. when walking on a flat surface? No Pain — Extreme Pain	PAIN1 ____
2. when going up or down stairs? No Pain — Extreme Pain	PAIN2 ____
3. at night while in bed? (that is - pain that disturbs your sleep) No Pain — Extreme Pain	PAIN3 ____
4. while sitting or lying down? No Pain — Extreme Pain	PAIN4 ____
5. while standing? No Pain — Extreme Pain	PAIN5 ____

Section B

STIFFNESS

Think about the stiffness (not pain) you felt in your_____ (study joint) caused by your arthritis during the last 48 hours.

Stiffness is a sensation of **decreased** ease in moving your joint.

(Please mark your answers with an " ✗ ".)

	Study Coordinator Use Only
6. How **severe** has your stiffness been **after you first woke up** in the morning? No Stiffness ——— Extreme Stiffness	STIFF6 ____
7. How **severe** has your stiffness been after sitting or lying down or while resting **later in the day**? No Stiffness ——— Extreme Stiffness	STIFF7 ____

Section C

DIFFICULTY PERFORMING DAILY ACTIVITIES

Think about the difficulty you had in doing the following daily physical activities caused by your arthritis in your _____ (study joint) during the last 48 hours. By this we mean **your ability to move around and take care of yourself.** (Please mark your answers with an " ✗ ".)

QUESTION: **How much difficulty have you had . . .**	Study Coordinator Use Only
8. when going down the stairs? No Difficulty — Extreme Difficulty	PFTN8 _____
9. when going up the stairs? No Difficulty — Extreme Difficulty	PFTN9 _____
10. when getting up from a sitting position? No Difficulty — Extreme Difficulty	PFTN10 _____
11. while standing? No Difficulty — Extreme Difficulty	PFTN11 _____
12. when bending to the floor? No Difficulty — Extreme Difficulty	PFTN12 _____
13. when walking on a flat surface? No Difficulty — Extreme Difficulty	PFTN13 _____

DIFFICULTY PERFORMING DAILY ACTIVITIES

Think about the difficulty you had in doing the following daily physical activities caused by your arthritis in your _____ (study joint) during the last 48 hours. By this we mean **your ability to move around and take care of yourself**. (Please mark your answers with an " ✗ ".)

QUESTION: **How much difficulty have you had . . .**	Study Coordinator Use Only
14. getting in or out of a car, or getting on or off a bus? No Difficulty — Extreme Difficulty	PFTN14 _____
15. while going shopping? No Difficulty — Extreme Difficulty	PFTN15 _____
16. when putting on your socks or panty hose or stockings? No Difficulty — Extreme Difficulty	PFTN16 _____
17. when getting out of bed? No Difficulty — Extreme Difficulty	PFTN17 _____
18. when taking off your socks or panty hose or stockings? No Difficulty — Extreme Difficulty	PFTN18 _____
19. while lying in bed? No Difficulty — Extreme Difficulty	PFTN19 _____

WOMAC VA3.1 QUESTIONNAIRE **WOM**C3-3

DIFFICULTY PERFORMING DAILY ACTIVITIES

Think about the difficulty you had in doing the following daily physical activities caused by your arthritis in your _____ (study joint) during the last 48 hours. By this we mean **your ability to move around and take care of yourself.** (Please mark your answers with an " ✗ ".)

QUESTION: **How much difficulty have you had . . .**	Study Coordinator Use Only
20. when getting in or out of the bathtub? No Difficulty — Extreme Difficulty	PFTN20 _____
21. while sitting? No Difficulty — Extreme Difficulty	PFTN21 _____
22. when getting on or off the toilet? No Difficulty — Extreme Difficulty	PFTN22 _____
23. while doing heavy household chores? No Difficulty — Extreme Difficulty	PFTN23 _____
24. while doing light household chores? No Difficulty — Extreme Difficulty	PFTN24 _____