Effects of Arginine on Osteoarthritis: A Pilot Clinical Trial

By

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ABSTRACT

Background: Osteoarthritis (OA) is a disease of high prevalence and economic burden, with yearly cost in Canada rising to $7.6 billion by 2031. It was recently discovered that arginine deficiency is associated with OA, suggesting arginine supplementation may be a novel nutraceutical for OA. We, therefore, undertook this phase IV, randomized, controlled and open-label two-arm clinical trial to assess the effects of arginine supplementation on cartilage degradation related gene expressions.

Patients and Methods: Primary knee OA patients who were scheduled to undergo total knee replacement (TKR) in six months were approached and recruited into the study. The consented patients were then randomly assigned into two groups - a control (no supplementation) or treatment (arginine supplementation for six months). The patients in the treatment group were provided and instructed to take 1.5g of oral L-arginine supplements daily until their TKR. All study participants were interviewed at the rheumatology clinic at St. Clare's Mercy Hospital and comprehensive questionnaire data were collected along with their blood samples. At their TKR surgery, joint tissue samples including cartilage, subchondral bone, and synovial membrane were collected with flash frozen method and stored at -80°C freezer until analysis. RNA was extracted from cartilage samples in LN2 environment with a Freezer Mill and five cartilage homeostasis related gene expressions including two cartilage matrix synthesis (*COL2A1* and *ACAN*) and three cartilage degradation enzymes (*MMP13*, *CTSK*, and *CTSB*) were assessed by real time PCR. Appropriate parametric tests like T-test and paired t-test and non-parametric tests like Chi square and Mann Whitney tests were used to compare

differences between treatment and control groups for gene expressions. WOMAC and SF-36 questionnaire was used to assess the health status and quality of life between the two groups at different time points.

Results: In total, we recruited 48 patients, ten patients were dropped out, one was rheumatoid arthritis, and one patient had no tibial sample. The final analysis included 36 participants (n=24 control, n=12 arginine supplementation) who completed the study. There was no significant difference in age, sex, BMI, and WOMAC scores between the two groups. The arginine concentrations were lower before the trial in both groups and increased by 23.5% and 33.5% in arginine and control groups after the trial, respectively. The mean time interval that patients taking arginine was 179 days ± 150 days. RNA was extracted from affected tibial cartilage tissue and cDNA synthesis was completed. Realtime PCR was completed to check the gene expression levels of *MMP13, CTSB, CTSK, ACAN* and *COL2A1*. There was no statistical difference in the gene expression of the genes of interest between the two groups. For WOMAC and SF-36 scores, at 6 months and 12 months, both groups showed significant improvements in all measures compared to baseline, indicating an improvement in knee pain, stiffness, function, physical and mental function of the individuals. This could be explained primarily due to the surgery itself which might have had an impact on the health status of the participants.

Conclusion: Our data did not show significant differences in cartilage synthesis and degradation genes between arginine supplementation and non-supplementation, which might be due to the small sample size. Further studies with a larger sample size are required to verify our findings.

GENERAL SUMMARY

The study was to determine whether arginine supplementation would affect cartilage degradation-related gene expressions in primary knee osteoarthritis (OA) patients. Knee OA patients scheduled to have total knee replacement surgery were randomly allocated into two groups: control and treatment, with the treatment group receiving arginine supplementation for six months before their surgery. RNA was extracted from cartilage tissue samples collected during their surgery, and five relevant gene expressions were assessed through real-time PCR (MMP13, CTSK, CTSB, ACAN, COL2A1). Thirty-six participants completed the study, with no significant difference in gene expression found between the two groups, although the arginine group had higher MMP13 expression. The study suggests that arginine supplementation may not have a significant effect in reducing cartilage degradation or influencing gene expression. However, due to the small sample size, further studies with larger sample sizes are necessary to confirm the results.

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ACAN	Aggrecan
AAOS	American Academy of Orthopedic Surgeons
ACR	American College of Rheumatology
AGC1	Mitochondrial aspartate-glutamate transporter
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthase
BMI	Body mass index
BP	Bodily pain
CRP	C–reactive protein
CRTM	Cartilage matrix protein
CRP	C–reactive protein
CTSCK	Cathepsin K
CTSB	Cathepsin B
CT	Computed tomography
cDNA	Complementary DNA
DM1	Type 1 diabetes
DM2	Type 1 diabetes
DNA	Deoxyribonucleic acid
DMOADs	Disease-modifying osteoarthritis drugs
EDTA	Ethylenediaminetetraacetic acid
ER alpha	Estrogen receptor 1 alpha
ESR	Erythrocyte sedimentation rate
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	General health perception
GTC	Guanidine thiocyanate
HRQL	Health-Related Quality of Life
IGF-1	Insulin like growth factor 1
IL-1β	Interleukin-1β
JSN	Joint space narrowing
K-L	Kellgren and Lawrence
LC-MS	Liquid chromatography–mass spectrometry
LK	Left knee
LN2	Liquid nitrogen
lysoPCs	Lysophosphatidylcholines
MCS	Mental Component Summary
MCID	Minimal clinically important difference
MCS	Mental Component Summary
MMP13	Matrix metallopeptidase 13
MRI	Magnetic resonance imaging
NDA	New Drug Application
NO	Nitric oxide

NSAIDs	Non-steroidal anti-inflammatory drugs
NTC	No template control
NFOAS	The Newfoundland Osteoarthritis Study
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PCR	Polymerase chain reaction
PF	Physical functioning
PHEIC	Public health emergency of international concern
PROMs	Patient Reported Outcome Measures
RK	Right knee
RNA	Ribonucleic acid
RP	Role limitation owing to physical health problems
RP	Role limitation owing to physical health problems
RT	Room temperature
SF	Social functioning
SF-36	Short form 36
siRNA	Small interfering RNA
TKR	Total knee replacement
TJR	Total joint replacement
THR	Total hip replacement
TGF beta	Transforming growth factor beta
TKR	Total knee replacement
TMIC	The Metabolomics Innovation Centre
TNF-α	Tumor necrosis factor-alpha
VDR	Vitamin D receptor
VT	Vitality
WHO	World Health Organization
WOMAC	Western Ontario McMaster Universities Osteoarthritis Index

1. INTRODUCTION

1.1 Osteoarthritis

1.1.1 Definition and characteristics

Osteoarthritis (OA) is a chronic multifactorial degenerative joint disease characterized by degradation of cartilage, synovial inflammation, osteophyte (bone spur) growth, subchondral bone remodeling, degeneration of ligaments and hypertrophy of the affected joint [1]. It affects approximately 300 million adults and is the main reason for joint pain and functional impairment worldwide [2]. It is usually multifactorial and a slowly progressing degradative process, destroying articular cartilage and other joint tissues [3]. Previously, OA was thought to be non-inflammatory, but it has been established that low-grade inflammation is crucial for disease pathogenesis. Even though OA's low-grade inflammation may not be the leading cause of the disease, it may still be a factor in disease development [4]. OA is more common among people with other chronic conditions, such as heart disease, diabetes, and obesity [5]. It affects all the joints of the body, most commonly the knee, hip, spine, and hand.

Until the 18th century, OA was grouped with rheumatoid arthritis. This caused confusion regarding the recognition of the disease and its nomenclature. Later, Alfred Baring Garrod, in 1859, separated the two diseases, naming one as rheumatoid arthritis. In 1904, John Kent Spencer termed the other disease OA after Joel E Goldthwaite differentiated the two diseases after confirmation from experts in this field. [6]

The Osteoarthritis Research Society International (OARSI) defines OA as "A disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation, and loss of normal joint function), that can culminate in illness." [7].

1.1.2 Types

There are two kinds of OA: primary OA and secondary OA. In both primary and secondary OA, there is a deterioration of joint cartilage, which leads to bones rubbing against one another. Sometimes, because of cartilage degradation, bones develop abnormal spurs. As a result, joints may become swollen, painful, and stiff [8]. Primary osteoarthritis results from the loss of articular cartilage and other joint tissues without any apparent underlying cause, usually from general wear and tear of joint tissue due to aging. As a result, it begins to manifest in people aged 55 and above. Everyone theoretically faces cartilage deterioration as they age, but some cases are more severe than others [8].

Secondary OA results from a specific trigger that exacerbates cartilage breakdown, like predisposing condition that has adversely altered the joint tissues (e.g., trauma to the articular cartilage or subchondral bone, infection, injury, or deformity) [9].

1.1.3 Symptoms and signs

OA primarily presents with joint pain, prompting patients to seek medical attention. Other symptoms include stiffness, deformity, and disability or loss of function [10 - 12]. It has been established that MRI-detected bone marrow lesions are related to pain in knee OA [13]. Clinically OA patients may reveal signs such as a restricted range of motion, tenderness at the joint, instability, crepitus, and muscle weakness (Table 1). Loss of function in the damaged joint may cause limited movement and difficulty performing daily tasks.

Table 1: Clinical Signs of OA

Reduced range of movement (Pain of range of movement)
Tenderness over the joint line
Muscle atrophy and weakness
Crepitus
Bony enlargement of the joint
Antalgic gait
Angular deformity (valgus/varus)
Trendelenburg test positive
Joint effusion
Fixed flexion deformity
Instability of joint

Radiologically, OA can be diagnosed with four main features: joint space narrowing, osteophyte formation, subchondral cyst formation, and subchondral sclerosis. Osteophyte development and capsular thickening lead to a reduction in range of motion, while joint surface irregularities are the cause of crepitus. One of the defining characteristics of OA is a narrowing of the joint space, whereas osteophyte growth is the most recognizable trait. To repair and redistribute abnormal joint loading or as a response to the cytokines released during the OA process, osteophytes are formed by endochondral ossification at the junction where cartilage meets synovium or periosteum. Subchondral sclerosis occurs in the areas of stress in the subchondral bone. This results in the deposition of new bone on pre-existing trabeculae with callus formation, whereas subchondral cysts emerge between thickened subchondral trabeculae [14].

1.1.4 Joints involved

Different types of joint involvement, the time at which symptoms appear, the rate of progression, and the severity of the condition can all affect how OA presents clinically. The knee, hip, hand, spine, and foot joints are commonly affected joints. Although OA can affect every synovial joint in the body, the knees and hips are the most frequently affected [15]. Joints that experience repetitive stress or injury are most susceptible to OA [16]. The knee joint is the most common lower-limb joint affected by OA. Patients suffering from OA experience knee pain when doing routine activities like walking and climbing stairs. Hip OA is difficult to diagnose as some people feel pain in different sites, like the groin area, buttocks, front of the thighs, side of the hips, or lower back. Patients suffering from

hip OA usually have difficulty with movements like sitting or getting up, or working standing for a prolonged period.

Hand OA usually occurs in people with a family predisposition to the condition, same as knee and hip OA with varied heritability estimates. It includes symptoms like pain and swelling of the joints in the hand and fingers. Pain is most experienced at the base of the thumb, which worsens with gripping and pinching movements. The prevalence of radiographic hand OA ranges from 27% -80% [17]. The ankle and joints within the foot are also affected by OA. The common joint affected on foot is the joint at the base of the big toe. This can result in pain with swelling or deformity at the joint and even formations of bunions. OA also commonly affects the spine, typically with stiffness or pain. Even though back pain is a common symptom in patients, diagnosing it as spine OA is challenging as back pain can be due to a joint problem or a disc or injury-related problem [16].

1.1.5 Prevalence

OA is the most prevalent debilitating condition and is now recognized as a serious public health issue by the World Health Organization (WHO). It is known to be one of the most common forms of arthritis, with over 300 million people and about 30% of the world population aged 50 or older affected by OA worldwide. [18 - 20]. OA affects approximately 9.6% of men and 18% of women globally [21]. OA is estimated to be the most significant cause of disability worldwide by 2030 [22]. One in four Canadians is expected to be living with arthritis by 2035 [23].

In Canada, OA is responsible for over 80% of hip replacement surgery and 90% of knee replacement surgeries [24] representing a significant economic burden matching cardiovascular disease [25]. OA imposes a tremendous economic burden on society [26] resulting in direct costs of about $7.6 billion in Canada by 2031 - a 2.6-fold increase from 2011 [27].

95,000 individuals have been reported to have arthritis in Newfoundland and Labrador, Canada, accounting for 22% of the population, higher than the national average of 16%. By 2036, this number is anticipated to rise from 95,000 to 127,000 [28]. OA can considerably restrict mobility; 38% of Newfoundlanders report arthritic pain impeding activities, in contrast to only 12% of people with other chronic diseases (individuals aged 45 years and older) [29] [23].

1.1.6 Risk factors

The etiology of OA is still unknown; it is usually multifactorial with various mechanical, metabolic, and inflammatory causes. OA is caused by non-modifiable (age, gender, and genetics) and modifiable risk factors (diet, obesity, injury or overuse, and abnormal loading of the joints). Age, genetics, overweight, repetitive strain injuries to the knee, bone density, muscle weakness, and joint laxity all contribute to the development of OA [30].

1.1.6.1 Age

Age is a major risk factor for OA. Age significantly increases the prevalence and incidence of radiographically and clinically diagnosed OA [17] [31]. With increasing age, there are

significant structural and mechanical changes, and changes in the matrix composition of articular cartilage. Articular cartilage undergoes age-related changes that increase the risk of its degeneration, that in turn causes the clinical syndrome of OA. These changes affect the attempts to regenerate or repair articular cartilage [30], [32]. Aged individuals have more brittle cartilage and chondrocytes with less anabolic and catabolic activity [33]. They also have a loss of the normal bone structure, increased stiffness of ligaments and tendons, and meniscal degeneration, which ultimately affect the joint tissues, leading to musculoskeletal aging, thus increasing the susceptibility to OA [33].

1.1.6.2 Sex

Sex is another important risk factor for the development of OA. Women are more prone than males to suffer from OA; in Canada, 13% of men and 20% of women reported having the disease in 2013. All women are at a 1.84 times higher risk of developing knee OA than men [24] [30]. Women are more likely than males to experience cartilage abnormalities or loss even when OA patients are not included, showing gender disparities in overall cartilage health before the onset of the disease [24]. For both men and women, hip OA prevalence rises simultaneously with age. In contrast, women's hip OA severity advances more quickly than men's [34]. Women experience a more significant age-related increase in the prevalence of knee OA [31]. The incidence of developing OA in women increases around menopause [35]. Hormonal factors are thought to affect the development of OA. However, there have not been any conclusive studies to prove that [36]. The difference between women and men may be due to bone loss, lack of muscle strength, and reduced cartilage volume [37].

1.1.6.3 Genetic factor

OA has a significant genetic component, varying from 40% to 65% depending on the affected joint site [38]. Much research, like the study of rare genetic disorders, epidemiological studies of family history, family clustering, and twin studies, have found evidence of a genetic influence on OA. Twin studies have demonstrated the influence of genetic factors around 60% in hip OA, between 65% in hand OA, 39% in knee OA, and 70% in OA of the spine [39]. The percentages suggest that the variation in susceptibility to developing OA in a population is likely by genetic factors [39]. It has also been found that genes regulating endochondral ossification, embryonic development, and postnatal skeletal maintenance are linked to OA[40]. A familial aggregation study conducted in the UK has estimated that the disease risk of a sibling of an individual with OA compared to the disease prevalence in the general population is ~5x [41]. OA may occur due to several gene interactions in cartilage, and bone. Studies have implicated linkages to OA, particularly on chromosomes 2q, 9q, 11q, and 16p. Like many common chronic diseases, OA is likely influenced by multiple genetic loci, each having a relatively small impact [42]. VDR, AGC1, IGF-1, ER alpha, TGF beta, CRTM (cartilage matrix protein), CRTL (cartilage link protein), and collagen II, IX, and XI were some genes linked in association studies with OA[39].

1.1.6.4 Obesity

Obese people are more likely to develop knee OA, 2.8 more times in males and 4.4 more times in females. With only ten pounds of additional weight, the force exerted on the knee increases by up to 60 pounds with each step [43][44]. Not all obese people develop OA, so

metabolic factors may also play a role. It has been found that individuals with excess body weight have been associated with a higher risk of hand OA [45]. Obese individuals are at a higher risk of developing knee OA than they are of developing type-2 diabetes or hypertension. Obesity is attributed to 27% of hip arthroplasty and 69% of knee arthroplasty cases [30]. The relationship between BMI and OA of the knee is linear; patients with higher BMI are at a higher risk of developing OA. Obesity alone or combined with metabolic syndrome raises the incidence of radiographic knee OA [46].

1.1.6.5 Joint injury

A joint injury such as trauma or repeated loading releases pro-inflammatory mediators like cytokines and chemokines, leading to widespread matrix degradation and loss. OA develops when cartilage degeneration exceeds the rate of chondrocyte remodeling. The risk of developing knee OA increases 3.86 times with injury or previous knee trauma [30]. Heavy physical workload, frequent exposure to several biomechanical stressors, such as bending of the knee, kneeling or squatting, standing for long hours (≥ 2 hours per day), walking ≥ 3 km/day, regular stair climbing, heavy lifting (≥ 10 kg), jumping, and vibration all are occupational risk factors to developing knee OA [47]. A British study showed that workers aged >55 years who were exposed to heavy weightlifting of more than 25 kg with climbing the stairs, or kneeling/squatting, had a five-fold increase in the risk of developing knee OA. Individuals who reported regular knee flexion without lifting were at 2.5 times greater risk [47]. Individuals participating in joint-intensive sports are also at a higher risk of developing OA [48]. Precautions, such as appropriate footwear and stretching and

strengthening exercises, can help reduce the onset and progression of OA in occupational and sports settings.

To lessen the burden of disease, non-modifiable risk factors should be taken into account, and modifiable risk factors should be addressed. Most people seek medical help once the disease has progressed to the point when substantial pain or decreased mobility interferes with daily activities. This makes it more challenging to identify the mechanisms causing OA. More research is required to understand this disease, its contributing variables, and how they interact in OA pathogenesis. Identification of risk factors, especially in the weight-bearing joints, and their management may reduce the likelihood of OA and avert pain and disability in the future[49].

1.1.7 Diagnosis

During a clinical visit, a patient of OA presents with significant signs and symptoms indicative of OA. A physical examination and radiographic findings such as X-ray is used to diagnose OA by a physician.

OA has been traditionally diagnosed using radiographs that show joint space width, osteophytes and the presence of subchondral bone abnormalities like cysts or sclerosis [50]. Plain radiography, like X-ray, has been the primary modality for diagnosing OA for decades. Recent studies on magnetic resonance imaging (MRI) have shown to be more accurate in diagnosing OA as plain radiography has a limited ability to detect osteoarthritic features at an early stage of the disease [51]. MRI provides soft tissue details with direct

visualization of the cartilage, allowing the joint to be assessed as a whole organ [52]. Furthermore, it enables multiplanar tomographic imaging, which enables the evaluation of three-dimensional structures and prevents the superimposition of overlapping structures. Both computed tomography (CT) scans and MRIs are used to investigate the structural progression of OA and links to the symptomatic severity [13]. MRI manipulates image contrast to highlight different types of tissues [50]. MRI can help show the thickness changes in cartilage over time in different affected joints in patients with OA, but it is a costly imaging technique and rarely indicates the acute stage of the disease [53]. Diagnosing early OA is challenging on MRI, and no gold standard has been established yet [54].

An invasive technique like arthroscopy can also reach a more significant portion of the synovium to detect knee joint changes, but it's challenging to perform [53].

For epidemiological studies, radiological diagnosis and grading have been the gold standard [55]. The system Kellgren and Lawrence (K-L grade) developed in 1957 is a scoring system that assesses radiographs for the presence and severity of individual radiographic features in patients with OA. This system grades the joint characteristics to assess OA severity based on osteophyte growth and joint space narrowing, on a scale from 0 to 4, with 0 being the best and 4 being the worst. A grade 2 is typically thought to represent radiographic OA, denoting the presence of definite osteophytes with joint space narrowing (Table 2) [55].

Table 2: The Kellgren-Lawrence System for Classification of Osteoarthritis

Grade 0	No radiographic features of OA
Grade 1	Doubtful narrowing of joint space, possible osteophytic growth
Grade 2	Possible joint space narrowing and definite osteophyte
Grade 3	Definite narrowing of joint space, multiple moderate osteophytes and minor sclerosis, and possible deformity of bone contour
Grade 4	Marked joint space narrowing, large osteophytes, severe sclerosis, and definite deformity of bone contour

Another scoring system commonly used is the Osteoarthritis Research Society International (OARSI) atlas. Joint space narrowing and osteophyte growth are evaluated separately and then combined for a final grade from a scale of 0 to 3 [56][57]. Out of three different criteria, one must be met; they are either a JSN grade of 2 or higher, sum osteophyte growth of grade 2 or higher, or JSN grade 1 combined with osteophyte grade1 and includes lateral and medial tibiofemoral compartments separately (49,50) when diagnosing radiographic OA, the OARSI atlas method provides more flexibility than the K-L system [56][57].

The American College of Rheumatology has developed another set of classification criteria for OA of the knee, hip, and hand (ACR) [58] [59] [60]. This criterion is based on the patient's history, physical examination, and laboratory or radiographic findings. The ACR criteria for hip and knee OA are listed in (Table 3) [59][61].The ACR criteria have been reported to have high sensitivity (89%) and specificity (91%) but poor reliability and cross-validity[62][63].

Table 3: American College of Rheumatology Criteria for classification of Hip and Knee Osteoarthritis (clinical and radiographic criteria)

Hip	**Knee**
Hip pain + At least 2 of the following three features	Knee pain + At least 1 of the three features +Osteophytes
-ESR <20 mm/hour	-Age >50 years
-Radiographic femoral or acetabular osteophytes	- Stiffness< 30 minutes
-Radiographic joint space narrowing (superior axial and/or medial)	- Crepitus

ESR: Erythrocyte sedimentation rate

Clinical history taking and examination of the joints by an expert clinician is not a substantial way to diagnose OA. Several validated scoring systems, or Patient Reported Outcome Measures (PROMs), are used to quantify the disease severity and outcome according to the patient's perspective. Having outcome measurements that can also reliably determine if changes have happened is essential for evaluating the efficacy of the treatment provided by healthcare professionals and researchers. Short form 36, Oxford Hip and Knee scores [64] and Western Ontario McMaster Universities Osteoarthritis Index (WOMAC) are a few examples of such assessment tools [65][64].

The WOMAC is designed primarily to assess hip and knee OA [66]. For assessing pain, disability, and joint stiffness for knee OA, the WOMAC is provided to OA patients [65]. This instrument was developed to evaluate clinically important patient-relevant changes in health status as a result of treatment intervention. It is self-administered and the most commonly used clinical tool for assessing patients with knee OA. It includes a total of 24

questions, with three subscales: pain (5 questions), stiffness (2 questions), and physical function (17 questions) (Table 4). The scores within the subscales can vary, with pain ranging from 0 to 20 points; stiffness, 0 to 8 points; and physical function, 0 to 68 points. Higher scores indicate worse pain, stiffness, and functional limitations. Adding all three subscale scores can also calculate a total score [66].

Table 4: Three subscales consisting of the 24 questions of the WOMAC questionnaire

Pain (5 questions)	Stiffness (2 questions)	Physical function (17 questions)
• Walking on a flat surface • Going up and down stairs • At night, while in bed • Sitting or lying • Standing upright	• After awakening • Later in the day	• Descending stairs • Ascending stairs • Rising from sitting • Putting on socks • Taking off socks • Bending to the floor • Lying in bed • Walking on a flat surface • Getting in/out of the bath • Standing • Getting in/out of the car • Getting on/off of the car • Getting on/off the toilet • Heavy domestic chores • Light domestic chores • Shopping

The Medical Outcomes Study 36-Item Short Form Health Survey (SF-36) is a well-known questionnaire designed to evaluate Health-Related Quality of Life (HRQL). It was standardized in 1990 as a self-report measure of functional health and well-being [67]. The latest version, SF-36 2.0, was published in 1996 with copywriting privileges to the Medical Outcomes Trust, Health Assessment Lab, and QualityMetric Incorporated. It has been used in various studies for chronic diseases, including OA, to give a brief yet comprehensive health status. The SF-36 measures eight scales: physical functioning (PF,10 items), role limitation owing to physical health problems (RP, 4 items), bodily pain (BP, 2 items), general health perception (GH, 5 items), vitality (VT, 4 items), social functioning (SF, 2 items), role limitation owing to emotional problems (RE, 3 items) and mental health (MH, 5 items). Two distinct concepts are measured using the SF-36: a physical component, represented by Physical Component Summary (PCS), and a mental component, represented by Mental Component Summary (MCS). All eight scales contribute to scoring the PCS and MCS measures (Table 5) [68].

Table 5: Two summary measures consisting of the eight scales of measures of the SF-36 form

Physical Component Scale (PCS)	Mental Component Scale (MCS)
Physical functioning	Vitality
Role limitation owing to physical health problems	Role limitation owing to emotional problems
Bodily pain	Social functioning
General health perception	Mental health

The scores range from 0 to 100, with higher scores indicating better health status. This questionnaire is sensitive to changes in health status and is reliable, valid, and responsive to changes in status. Good validity has been shown for patients with arthritis.

Laboratory tests like C–reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are often ordered to support a diagnosis of OA. Several recent reports show that ESR and CRP are slightly elevated in OA [69][70][71]. It was found in a study that the level of high-sensitive CRP was increased more in progressive knee OA than in non-progressive OA [72]. Mean CRP levels were higher in OA patients than in healthy individuals [30]. ESR and CRP were higher in patients with knee OA and were related to clinical features. ESR and CRP were significantly elevated in patients with tenderness, swelling, and patellar ballottement [73].

1.1.8 Treatment

As treatment options for OA are limited, it is advised to start a self-management program that includes a nutritious diet, regular exercise, and joint-protection exercises as soon as the condition is diagnosed and as it progresses. At an early stage, OA is usually undetected as patients notice symptoms of pain and discomfort at the end stage of OA development. Thus, the detection of OA at an early stage and its management will significantly reduce long-term damage.

1.1.8.1 Pharmacological therapy

Currently, there are no cures for OA. Currently, treatments only target symptomatic relief like pain or improvement of function. These include oral agents such as analgesics, non-steroidal anti-inflammatory drugs (NSAIDs) and opiates, intraarticular injections including steroids and hyaluronans, and physical therapies. Derivatives of glycosaminoglycans usually found in articular cartilage, like glucosamine sulfate and chondroitin sulfate, have been reported as effective analgesics for pain relief in OA [74][75][76].

A treatment guideline for OA provided by OARSI provides a valuable list of interventions that proved to be effective in improving pain in OA patients [77]. This guideline also provides non-pharmacological management strategies like weight loss, education, and intervention, exercise, physiotherapy, and other mechanical aids [78].

However, there is little evidence that these therapies affect the structural progression of OA, so many patients are eventually faced with joint aids as the only option to improve their quality of life.

Over the past 10 years, several drugs (disease-modifying OA drugs (DMOADs)) and nutraceuticals have been evaluated in clinical trials to determine whether they can halt or reverse the structural progression of OA. These include matrix metalloproteinases inhibitors, bisphosphonates, cytokine blockers, calcitonin, inducible nitric oxide synthase inhibitors, doxycycline, chondroitin, glucosamine, and diacerein, but the evidence is inconsistent or inconclusive. Despite major research efforts, there are currently no DMOADs approved by the Regulatory Authorities [79].

1.1.8.2 Surgical therapy

So far, total joint replacement therapy (TJR) is considered the most effective treatment for end-stage OA patients [80] where oral analgesics fail to alleviate symptoms in advanced OA. TJR is a surgery where surgeons remove worn-out cartilage to replace a damaged joint with an artificial joint made of metal, ceramic, or plastic [81]. The most common diagnosis for primary hip and knee replacement patients was OA, at 69.4% and 99.3%, respectively [82]. With the number of TJR surgeries steadily increasing in Canada, more than 75,000 total knee arthroplasty (TKA), and 60,000 total hip replacements (THR) are performed yearly [83], so finding novel therapeutic agents is crucial. TJR surgeries can considerably improve patients' quality of life, although this improvement might be limited to a short period [84].

Many patients do not meet the minimal clinically important difference (MCID), a patient-reported measure of the minimal acceptable improvement in the patient's symptoms for joint pain and/or function improvement, even though most patients experience symptomatic improvement after TJR. A systematic review of 14 studies found that up to one-third of patients receiving TKR and one-quarter undergoing THR reported unfavorable long-term pain outcomes. Estimates of the percentage of patients who do not reach the MCID vary [85]. TJR surgeries may not be a successful therapeutic option due to prolonged recovery durations, patients' anxiety over complications and anesthesia, and financial difficulties [86]. The long wait times for arthroplasty in Canada can further complicate this as a treatment choice [87].

As the pathogenesis of OA remains elusive, it is difficult to diagnose this disease at an early stage. Early diagnosis of OA would allow early and targeted treatments, thus preventing the progression of the disease or having joint replacement surgeries. This would help recover major societal and economic costs and better quality of life for the patients by reducing pain[88][89][90].

Current treatments of OA only target the symptoms of OA without modifying the disease to restore the changes caused by the disease. Analgesics are merely a symptomatic therapy option; they do not improve the lubricating and compressive properties of the remaining cartilage or help replace missing cartilage [3]. Changes in lifestyle and activities may not have much of an impact by the time a patient has significant pain and discomfort to seek medical attention. Hence, a valid, non-invasive method would be significantly helpful in patients with OA.

There is still an inconclusive agreement between clinicians regarding treatment for OA. Even though there has been enough literature documenting the positive effect of intra-articular hyaluronic knee injections [91]. The American Academy of Orthopedic Surgeons (AAOS) does not recommend the use of hyaluronic acid for knee OA patients because of inconclusive clinical evidence [92].

Continued research in Human genetics holds the promise of refining disease diagnosis and tailoring precise treatments for specific conditions, thereby enhancing our ability to effectively combat a wide range of diseases.

Metabolic research plays an important role in uncovering innovative treatment options for various medical conditions. By exploring into the intricate processes that regulate metabolism, researchers gain critical insights into the underlying mechanisms of diseases. This knowledge is important in developing targeted interventions, potentially developing ways we can approach treatments and therapies for diseases. Additionally, metabolic research contributes to a deeper understanding of personalized medicine, paving the way for tailored and more effective healthcare solutions for individuals with diverse metabolic profiles.

1.2 Metabolomics of OA

OA treatment is currently inadequate and limited to pain management and entirely removing the joint. A disease-modifying drug for OA is yet to be found [93]. There are no known disease-modifying agents that have been put into clinical use. Metabolomics research has the potential to identify disease-modifying agents. A thorough understanding of the disease status of these joint tissues is necessary to develop novel therapeutic approaches that target the osteoarthritic degradative and inflammatory processes in cartilage, synovium, or bone. Findings from a metabolomic analysis will provide new information about the pathogenesis of OA and help to develop personalized tools for managing OA towards reducing the social and economic burden and improving the quality of life for OA patients.

The latest and rapidly developing tool, metabolomics, has been found to be one of the most comprehensive and reliable tools for examining physiological status, finding new

biomarkers, and analyzing metabolic pathways [94]. Metabolites are intermediate and end products of various cellular processes. Their concentration levels serve as a good indicator of a sequence of biological systems in response to genetic and environmental influences. Different body fluid metabolites and diverse cells can be measured using metabolomic analysis, helping improve our knowledge about the mechanism and underlying metabolism at a molecular level corresponding to various human traits and diseases [95]. Metabolomics provides a snapshot of the entire physiology of the host and its response to the environment and genetics, which can later be associated with the outcome phenotype and endotypes. Biological fluids such as plasma, urine, saliva, cells, and tissue extracts are a few of the many different samples used for metabolomic analyses.

So far, studies on metabolomics have discovered markers linked to various diseases like cancer [96] , Alzheimer's disease [97] , cardiovascular diseases [98], and diabetes [99]. In recent years, metabolomics has emerged as a powerful tool for studying the metabolic changes that occur in OA. Metabolic changes in OA can be identified with the changes in different metabolites and their pathways.

By analyzing metabolites in biological samples, metabolomics can provide insights into the dysregulated biochemical pathways in OA. Chondrocytes, the cells responsible for cartilage development and maintenance, rely on anaerobic metabolism and glucose as their primary energy source. Several studies have explored the association between plasma glucose concentration and OA. A clinical and epidemiological survey showed that symptomatic OA patients had significantly higher plasma glucose concentrations

compared to non-OA controls [100]. This finding suggests a potential link between hyperglycemia and OA. Further investigations have provided additional evidence supporting the association between hyperglycemia-related disorders, such as diabetes, and OA. In a study involving 6,197 participants, an increase in fasting glucose concentrations by 0.85 mmol/l was associated with an 18% higher risk of hand OA in men [101]. This finding suggests that elevated glucose levels may contribute to the development of hand OA.

Moreover, a population-based longitudinal study with a follow-up period of 20 years with a sample size of 927 revealed that type 2 diabetes (DM2) was associated with 2.1 times increased risk of total joint replacement (TJR) [102]. Notably, the risk of getting TJR increased with the duration of diabetes. More recently, a study with a large sample size involving 37,353 patients with type 1 diabetes (DM1) and 1,218,254 patients with DM2 found significant associations between diabetes and knee OA. Specifically, DM1 was associated with 1.4 times increased risk of knee OA, while DM2 was associated with 2.75 times increased risk of knee OA [103]. These findings highlight the potential impact of DM1 and DM2 on the development of knee OA.

In summary, research suggests that elevated plasma glucose concentration and hyperglycemia-related disorders, such as diabetes, are associated with an increased risk of OA. This evidence indicate that higher glucose levels may contribute to the development and progression of OA.

The application of metabolomics on OA research has also identified several promising and potential clinically actionable metabolic markers [104]. A novel metabolic marker – the lysophosphatidylcholines (lysoPCs) to phosphotidylcholines (PCs) ratio was found to be associated with advanced knee OA [105]. Similar metabolites, phosphatidylcholine acyl-alkyl C34:3 and phosphatidylcholine acyl-alkyl C36:3, were also identified to be associated with OA and diabetes (p-value <0.003) [106]. Moreover, the study found that diabetic patients exhibited reduced concentrations of these two PCs compared to controls. These findings suggest that alterations in phosphatidylcholine metabolism occur in OA, and the same metabolic pathway is shared with metabolic-related diseases such as diabetes.

Furthermore, since OA is a multifactorial and heterogeneous condition, metabolomics has the potential to assist in classifying OA patients into distinct subtypes. This classification would enable the development of more targeted and personalized interventions specific to each subtype. Tailoring treatments to OA patients' unique metabolic profiles would improve interventions' effectiveness and ultimately enhance patient outcomes. Recently, a metabolomic approach was used in a study to identify distinct subgroups which might help unravel the pathogenesis and develop targeted therapies for OA It was determined that there are three metabolically different subgroups of OA, probably caused by variations in the metabolism of carnitine, lipids, and collagen [107].

In a new study, The Newfoundland Osteoarthritis Study (NFOAS), endotypes of OA patients were also identified using metabolomic analyses [108]. It demonstrated three endotypes in OA, characterized by C4, arginine, and lysophosphatidylcholine levels in

plasma. This resulted in primary OA patients being classified as having muscle weakness, arginine deficiency, and low inflammatory OA [104]. This will enable the identification of additional subgroups within OA patients, each characterized by distinct traits, allowing for more targeted and personalized treatment approaches.

Several novel metabolic ratios associated with pain and function non-responders to total joint replacement (TJR), based on categorization by WOMAC and MCID criteria, have also been identified using metabolomic analysis[85].The metabolite ratios and metabolites identified were considered novel predictors for TJR outcome measures. They suggested their roles for muscle breakdown in function non-responders to TJR and also in inflammation in both pain and function non-responders to TJR [85]. Such findings would help develop tools to identify patients who will or will not benefit from surgery. This would not only spare patients the stress of invasive procedures but also enable physicians to focus more on pain management strategies, ultimately reducing the economic burden associated with these surgeries.

The metabolomic studies conducted on OA have yielded promising results. By analyzing the metabolites in biological samples, metabolomics can provide insights into the dysregulated biochemical pathways in OA. While there is still much to be understood about the molecular mechanisms underlying OA, metabolomics can identify potential biomarkers of OA and guide the development of new therapeutic interventions for this debilitating disease. Further studies are needed to confirm these results and to determine the optimal strategies for implementing metabolomics in the clinical management of OA.

Findings from metabolomic analyses provide new information about OA's pathogenesis and help develop personalized tools for managing OA towards reducing the social and economic burden and improving the quality of life for OA patients. Recently there has been a rise in interest in nutraceutical supplements, which contain a diverse class of molecules that can promote cartilage production and significantly reduce inflammation, oxidative stress, discomfort, and stiffness of joints [2].

Extensive evidence gathered in cellular OA models, animals, and human RCTs, has shown supplementing with nutraceuticals to be a key adjuvant technique in the management of OA [2]. Pharmacological analgesics have limited efficacy; they may also be associated with significant side effects, especially when used over an extended period. Growing data indicates that the effect of nutraceuticals on OA pain may be related to their anti-inflammatory properties, even though the precise molecular mechanism underlying it is unknown and poorly understood [109].

Recently a meta-analysis of 42 random clinical trials (RCTs) utilized nutraceuticals like chondroitin sulfate, glucosamine sulfate, collagen, and hyaluronic acid and found improvements in all OA measurement parameters expressed through the total WOMAC index [110]. The nutraceuticals that showed significant improvements in clinical symptoms and decreased inflammatory index in patients with OA are chondroitin sulfate, glucosamine sulfate, collagen, hyaluronic acid, and methylsulfonyl [111]. Even though enough studies have shown the beneficial effect of nutraceutical usage in OA, there is

little statistical significance, and some side effects that have kept them from mainstream medical use.

In conjunction with traditional therapy, nutraceuticals may be a viable management method for OA. Studies with big sample sizes are urgently needed before this unconventional approach can be considered definitively in clinical practice to test the efficacy and safety of these treatments over long periods. To our knowledge, data on the metabolomics study of a nutraceutical, arginine, and OA are still sparse. This novel nutraceutical needs further exploration in OA patients to test its efficacy.

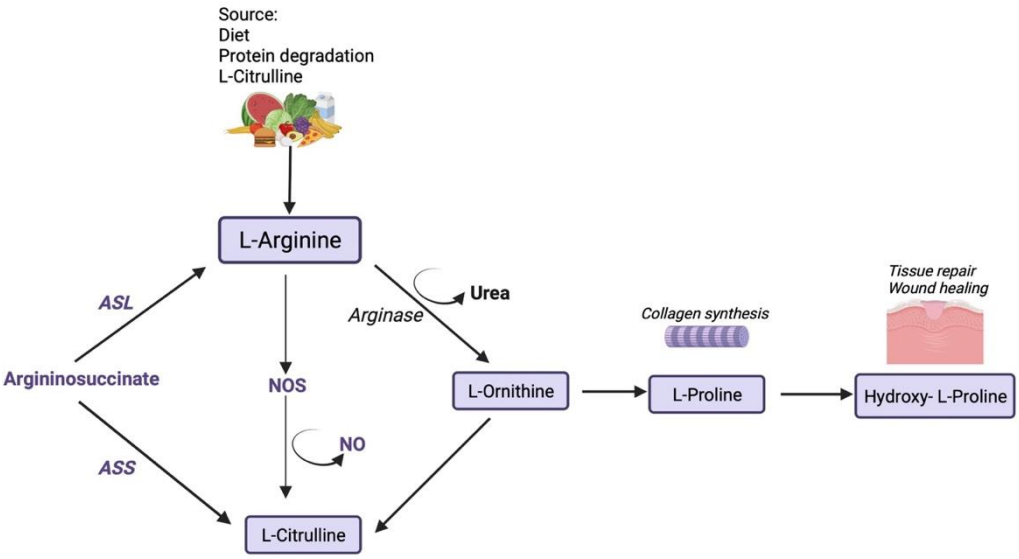
1.3 Arginine

Arginine is a semi-essential amino acid involved in various physiological processes, such as protein synthesis, wound healing, and immune function. It has been shown to possess anti-inflammatory and anti-oxidative properties that may help reduce the inflammation and oxidative stress associated with OA. Additionally, arginine has been reported to enhance the production of nitric oxide, a vasodilator that plays a crucial role in maintaining the health of joints.

L-arginine was identified as a precursor for NO in 1980(53), along with many other molecules, including urea, proline, glutamate, creatine, and agmatine [112]. There is widespread interest in arginine in various studies because it engages in multiple metabolic pathways within the human body that play important roles in various physiological and pathophysiological conditions [113]. Because humans need an additional intake of dietary

arginine, like during development during infancy, pregnancy, severe immunological challenges, or burn injuries [114][115], arginine is classified as a semi-essential amino acid. Adult humans can synthesize arginine from glutamine, glutamate, and proline, but the majority of circulatory arginine comes from dietary amino acids.

The source of arginine is from dietary protein intake, body protein breakdown, or endogenous *de novo* arginine production (Figure 1). Around 10–15% of whole-body arginine production is contributed by *de novo* arginine production due to the conversion of citrulline to arginine, catalyzed by the enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). This conversion is part of the intestinal-renal axis, with intestinal production of citrulline and renal synthesis of arginine [116]. Due to the relatively



high arginase activity in the intestinal mucosa, around 40% of dietary arginine is typically extracted in the splanchnic area [117].

Figure 1: Arginine metabolic pathway. ASL:Argininosuccinate lyase, ASS:Argininosuccinate synthase.

It is reported that in humans, the normal range of blood arginine is 21–137 μM [100], and the daily dietary intake of arginine is about 4–6 g [118][119]. Ultimately, about 50% of dietary arginine enters the circulatory system.

Citrulline, an immediate precursor of arginine, may be a limiting factor for the de novo production of arginine[120]. The enzymes arginosuccinate synthase (ASS), and arginosuccinate lyase (ASL) catalyzes the conversion of citrulline to arginine, which accounts for 10–15% of the overall body's arginine production under normal circumstances [121][122]. The urea and citrulline-NO cycles involve citrulline and the enzymes arginosuccinate synthase (ASS) and arginine succinate lyase (ASL), which are needed for two pathways for the endogenous *de novo* synthesis of L-arginine [120], [123]. The metabolic fate of arginine is determined by the distribution of arginine between intracellular transporters and arginine-converting enzymes and between arginine-converting and arginine-synthesizing enzymes [120]. Proline can also be made from arginine and can produce collagen, tissue repair, and wound healing. Proline is hydroxylated to hydroxyproline post translationally[120].

L-arginine is essential for NOS-dependent NO biosynthesis. Arginine levels may be reduced in the blood due to reducing *de novo* production and elevated arginase activity. This is reported in acute and chronic stress conditions, often characterized by NO synthase activity. Introducing supplementation of arginine or citrulline may influence *de novo* arginine production and NO metabolism by increasing substrate availability. There is considerable data that suggests arginine supplementation is beneficial for growth,

health, and disease prevention. It may also offer new and effective treatments for obesity, diabetes, and metabolic syndrome [124].

One of L-arginine's most extensively studied therapeutic applications is its potential to improve cardiovascular health. L-arginine is a precursor for NO, which has vasodilatory effects that can help to reduce blood pressure and improve blood flow. Several studies have investigated the effects of L-arginine supplementation on cardiovascular health with mixed results [125]. While some studies have reported improvements in endothelial function and blood pressure, others have found no significant effects[126] [125]

L-arginine has been shown to play a role in cutaneous wound healing by promoting collagen synthesis and angiogenesis [127][128]. Several studies have investigated the effects of L-arginine supplementation on wound healing with mixed results. While some studies have reported improvements in wound closure and healing time, others have found no significant effects [127][128].

L-arginine has been shown to play a role in immune function by promoting the proliferation and activation of immune cells. Several studies have investigated the effects of L-arginine supplementation on immune function, with mixed results. While some studies have reported improvements in immune function, others have found no significant effects.

In clinical research, arginine supplementation ranging from 6 to over 21 g/day (up to 8g per single dose) has been administered [129]. Single doses between 3 and 8 g seem to be

safe and rarely cause adverse reactions but single doses over 9 g, mainly when they are part of a dosing schedule of more than 30 g per day, have been linked to gastrointestinal discomfort, nausea, and (osmotic) diarrhea [120].

Research shows that arginine supplementation benefits health, disease, and growth and may offer cutting-edge and efficient treatments for obesity, diabetes, and metabolic syndrome. Patients with sickle cell disease suffering from pulmonary hypertension, preventing age-related glomerular injury, and improving wound healing had benefited from arginine supplementation. Arginine was also suggested as a treatment for hypertension to break the vicious cycle that maintains low NO levels[120]. Effects of arginine on patients with OA need to be explored to understand its effect on the pathophysiology of development and progression of OA.

1.4 Arginine and OA

Because of the recently published studies on OA, interest and progress in the field of metabolism in OA have increased. There have been studies describing the metabolic changes and associated pathways involved in the pathogenesis of OA in both human and animal models [130].

Studies on humans show that changes in amino acid metabolism may be significantly related to the etiology of OA. Different metabolites, including phospholipids and altered amino acids, arginine, and alanine, have been discovered as possible biomarkers to distinguish OA patients from healthy individuals[130]. Several studies have investigated

the effects of arginine on OA, both *in vitro* and *in vivo*. In a study conducted by Park *et al.* (2018), the authors investigated the effects of arginine on chondrocytes, the cells responsible for producing and maintaining cartilage. They found that arginine treatment significantly increased the proliferation and viability of chondrocytes and inhibited the production of inflammatory mediators, such as interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α). IL-1β and TNF-α–enhanced transcription of inflammatory genes such as iNOS and COX2 as well as matrix-degrading enzymes *MMP-3* and *MMP-13* and decreased collagen II, aggrecan [101]. In another study by Qian *et al.* (2019), the authors investigated the effects of arginine on OA in a rat model. They found that arginine treatment significantly reduced the severity of OA by decreasing cartilage degradation and reducing the production of pro-inflammatory cytokines, such as IL-1β and TNF [101].

It has been recently found that arginine concentration decreases in OA patients [131][132]. Studies in OA patients have shown that plasma arginine concentrations are significantly lower in the OA patients than in the healthy controls (53.55±16.37 vs. 70.20±25.68 μmol/l, $P<0.05$). Arginine concentrations were found higher in the synovial fluid than in plasma (76.96±16.73 vs. 53.55±16.73 μmol/l, $P<0.00001$). By increasing the activity of arginase, the inflammatory processes may decrease L-arginine levels by an enzyme that converts L-arginine into L-ornithine[133]. In an Italian study, arginine, in combination with other amino acids, was found to support cell division and the growth of osteoblasts [134]. Ornithine is a precursor for the synthesis of proline, which helps with collagen production. In plasma, the concentration of ornithine was found to be 2.2

times higher in knee OA patients than in controls ($P = 1.1 \times 10^{-22}$), with the ratio of arginine to ornithine lower in knee OA patients than that in controls ($P = 4.7 \times 10^{-48}$) [134]. This finding supports arginine depletion in OA due to over activity of arginine catabolism. It was further established when downstream arginine catabolism metabolites were significantly increased in OA patients than in controls[130].

Using a metabolomics approach, six metabolites were identified to be significantly associated with knee OA, of which arginine was the most significant metabolite ($P < 3.5 \times 10-13$). Plasma concentration of arginine in knee OA patients was, on average, 69 μM lower than that in non-OA controls. The overactivity of the arginine to the ornithine pathway caused an imbalance between cartilage repair and degradation, leading to the depletion of arginine in OA patients.

Taken together, the evidence suggests that arginine may have beneficial effects on the management of OA by reducing inflammation and oxidative stress, enhancing cartilage synthesis, and improving joint health. This finding is very promising. Therefore, we want to see the effect of arginine on a molecular level and hypothesize that the arginine supplement has a disease-modifying effect on human articular cartilage by regulating genes related to cartilage maintenance and repair. So, we undertook this pilot clinical trial to compare the effects of oral arginine supplementation vs. no supplements on cartilage gene expressions in patients scheduled to undergo total knee replacement surgery (TKR) due to primary OA in six months. If confirmed, arginine could be a promising therapeutic option for the management of OA.

2. Hypothesis and study rationale

We hypothesize that arginine supplement has disease-modifying effect on human articular cartilage by regulating genes related to cartilage maintenance and repair.

3. Objective

3.1 Primary outcome

Target gene expressions in osteoarthritic knee cartilage after at least 6 months of supplementation.

Target gene expressions in cartilage tissue obtained during TKR. Genes include cartilage degradation enzymes:

- *MMP13 (matrix metallopeptidase 13)*
- *CTSK (cathepsin K)*
- *CTSB (cathepsin B)* which we previously found over expressed in OA-affected cartilage [135]

Cartilage syntheses genes:

- *ACAN (aggrecan)*
- *CTXII (type II collagen),* which we previously found down expressed in OA-affected cartilage [135]

3.2 Secondary outcome

The severity of pain reduction (WOMAC scores), and improvement in mental and physical function (SF-36 scores).

4 Method

4.1 Study Design

This was a phase IV, randomized, controlled, and open-label 2-arm study to assess the effectiveness of oral L-arginine on advanced primary knee OA patients (aged 19 years and older) that were scheduled for TKR in six months. Group I (n=24): Subjects received 1.5g of L-arginine from week 0 to time of TKR. Group II (n=24): Subjects received nothing from week 0 to time of TKR (Figure 2). The trial was conducted at St. John's, Newfoundland, Canada. Ethical approval was obtained from the Health Research Ethics Authority of Newfoundland and Labrador (reference number is 2018.194). Patients received information regarding the study, including information about the risks and benefits of the procedure. They were given ample time to ask questions and were welcome to express their opinions anytime during the project. Then, written informed consent was obtained from all participants before enrolment. The trial is registered in the NCT database (NCT03665116).

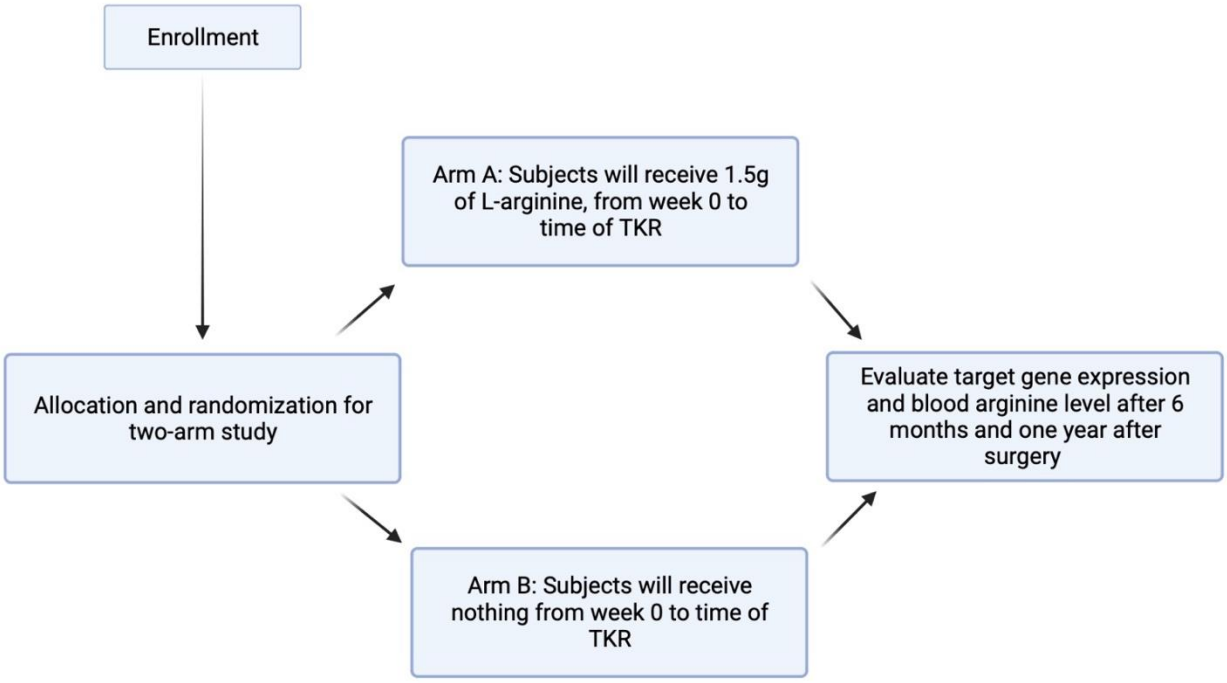


Figure 2: Allocation and randomization of the two arms of the study

4.2 Study Population

Patients with a history of primary knee OA scheduled to undergo TKR in six months, irrespective of their sex and gender, were considered for this prospective, randomized, open-enrollment pilot study. The participants who were enrolled in the study were selected from a population that lived in a 50-kilometer perimeter of St. John's, NL. Staff members contacted the patients at the Miller Center's Total Joint Assessment Centre (TJAC). They were then asked if they were interested in participating in the clinical trial. Compiled list of names with the phone numbers of the patients who agreed to take part in the clinical trial was forwarded to the research team.

4.3 Inclusion Criteria

Patients who were to undergo total knee replacement surgery in 6 months due to primary knee OA, and were ambulatory, were eligible for enrollment in the clinical trial. They were selected from individuals who lived within 50 kilometers of St. John's, the capital city of Newfoundland, and Labrador, Canada.

4.4 Exclusion Criteria

Patients were excluded from the study if they had a history of osteoporotic fracture, previous knee surgery, or arthroscopy within 6 months. Patients clinically diagnosed with secondary OA or inflammatory arthritis were excluded from the study. Patients were also excluded if they had a history of taking cod liver oil supplementation or supplementation containing arginine within 6 months. Patients who used bisphosphonates within 2 years or intra-articular viscosupplementation or platelet-rich plasma at any point were also excluded from the study.

Initially, 380 individuals were approached for the study after being screened to live in a 50km perimeter of St. John's, out of which 49 individuals agreed to participate (Figure 3). Of these 49 individuals, one individual was given a Vit C supplement and later excluded from the study; from the remaining 48 participants, 24 of them were selected to receive arginine, and other 24 of them were set as controls and were not given anything (Figure 3). The total response rate of the patients was 13%. A total of 48 knee OA patients aged $\geq$ 19 years were enrolled in the study. The patients on the list were approached via phone by a research assistant to explain the details of this study. Patients' verbal consent to screening

was obtained during this phone interview and recorded in the clinical trial database; patients were screened if they underwent TKR due to primary knee OA and were ambulatory. Eligible patients were then invited to a clinical visit at the Rheumatology Clinic in St. Clare's Mercy Hospital. The consent form and questionnaires to assess OA patients' mental and physical functions (General Questionnaire, WOMAC, and 36-Item Short Form Health Survey (SF-36) were mailed to them before their scheduled clinical visit. After they had read the consent form, the research assistant reached the patients by phone to obtain their verbal consent for further contact regarding participating in the study. Verbal consent status was recorded in the clinical trial database. At the clinical visit, patients were allowed to ask questions about the study, answered by a research nurse/assistant or one of the investigators, before signing the consent form. The consent form was signed by the patient, research nurse/assistant, and one of the investigators simultaneously, and the patient was given a copy of it as a record. Demographic information was collected by the research staff from the questionnaire provided to the participants. Data regarding the height and weight of the patients were retrieved from the general questionnaire, which was provided to the patients during enrollment. Body mass index (BMI) was calculated by dividing weight in kilograms by squared height in meters. Age was calculated by subtracting the date of birth at the time of the surgery. A qualified research nurse collected 6.5 ml of blood. WOMAC and SF-36 data and 6.5ml blood were collected during the pre-admission clinic visit right before their surgery. Cartilage tissue of the replaced joint, normally discarded, was collected during the surgery. Confirmatory diagnosis of OA was made based on the clinical judgment of the orthopedic surgeons and the American College of Rheumatology criteria. [59][136]. Phone interviews were conducted six and 12 months after the surgery to collect

post-surgery WOMAC and SF-36 data. A blood requisition form was mailed to the participants after the last phone interview, and 6.5 ml of blood was collected for the study during their next routine fasting blood work. A total of 48 participants were included in this study. The same assessment process was used for all candidates to confirm it is equitable (e.g., used standard tests, assessment questionnaires, and interview questions). For this project, we focused on meeting the eligibility criteria of patients suffering from knee OA, irrespective of their sex, gender, and ethnicity. Out of those 48 participants, 11 participants withdrew their consent at different stages of the trial (Table 6). One of the patients (CT019) had withdrawn their consent after 6 months post-op, so there were no follow-up questionnaires from this patient (all his data collected before that was used till his withdrawal). One individual had received a Vit C supplement, one individual was diagnosed as a patient with rheumatoid arthritis by the surgeon during the total knee replacement surgery, and one patient had only his femur samples collected (no tibial samples collected) (Figure 3). Therefore, the final analysis included 36 participants (n=24 receiving no supplementation and n=12 receiving arginine supplementation) (Figure 3). These 36 individuals were included for descriptive analysis: age, sex, BMI, and WOMAC score (at baseline) for pain, stiffness, and functional deficit and total WOMAC score (at baseline).

Table 6: Participants who withdrew their consent with reason.

Study ID	Reason
CT012	She stopped taking the pills as she thought they induced stress.
CT014	She changed her mind and stopped taking the pills on her own.
CT016	He was not taking arginine tablets regularly.
CT017	The arginine tablets were too big to swallow for the patient.
CT019	Moved to another province.
CT021	The patient was disappointed during the initial appointment that she was assigned to the arginine group and was nervous about taking the tablets.
CT028	The physician informed the patient that he wouldn't be having TKR surgery because of his existing conditions and the surgeries he'd had in the past, it would be too painful for him to have the TKR, and he won't benefit from it.
CT029	The patient's stomach hurt severely after taking the tablets.
CT035	The patient found no benefit from the pills.
CT041	The patient had been experiencing congestion and cold-like symptoms since starting the tablet.
CT044	She was experiencing constipation from arginine tablets.

Note: All the 11 participants who withdrew their consent were from the treatment (arginine) group.

4.5 Patient disposition flowchart

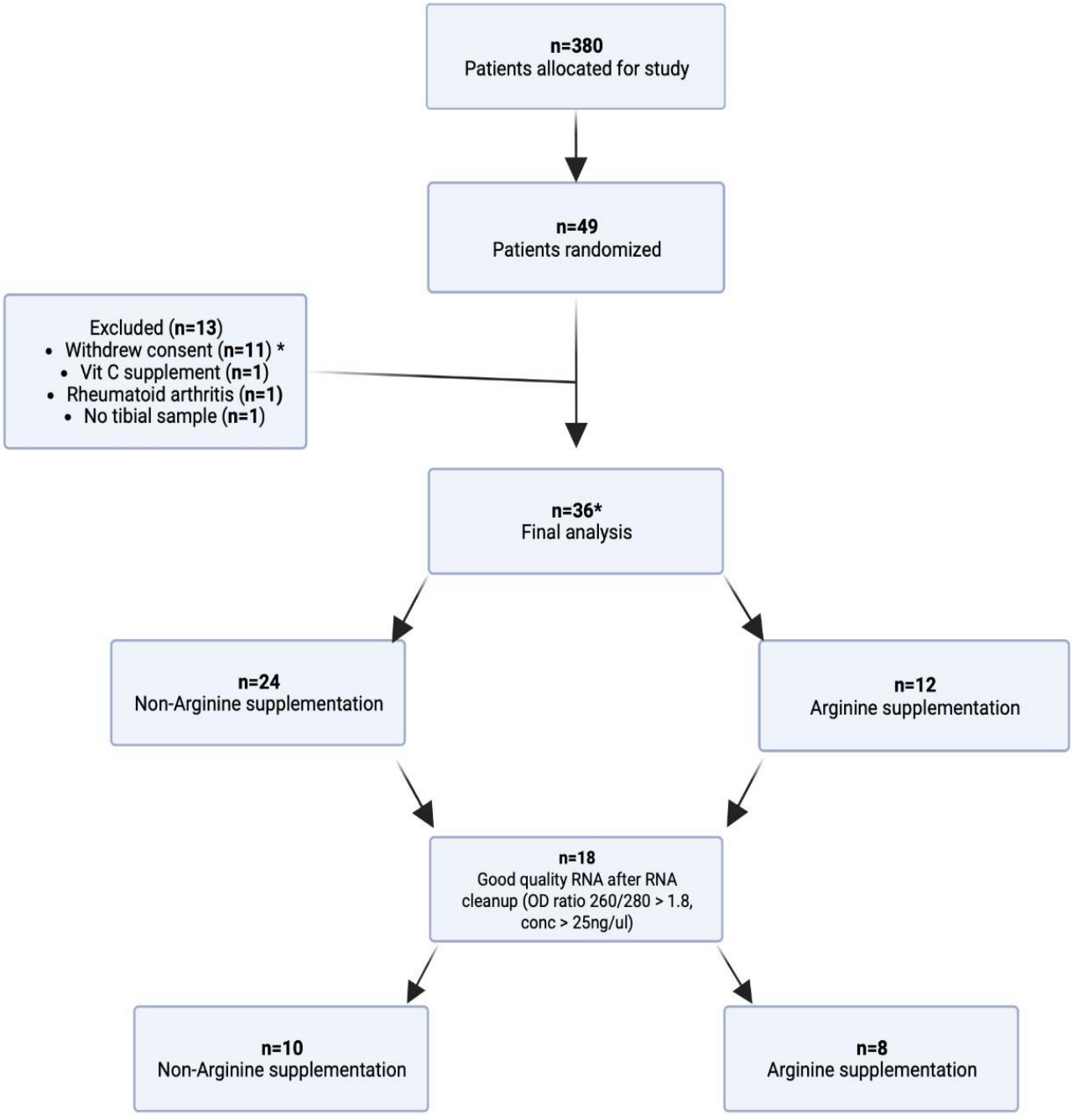


Figure 3: Patient disposition flowchart. * Including study ID CT019

Out of those 36 individuals, 24 individuals (n=9 receiving arginine supplementation and n=16 receiving no supplementation) blood sample was sent out to measure their arginine concentration in blood (Figure 4). Patient ID CT002 had surgery on both of her knees. So, data from both the right and left knee were used for the study and counted separately as two samples for further analysis.

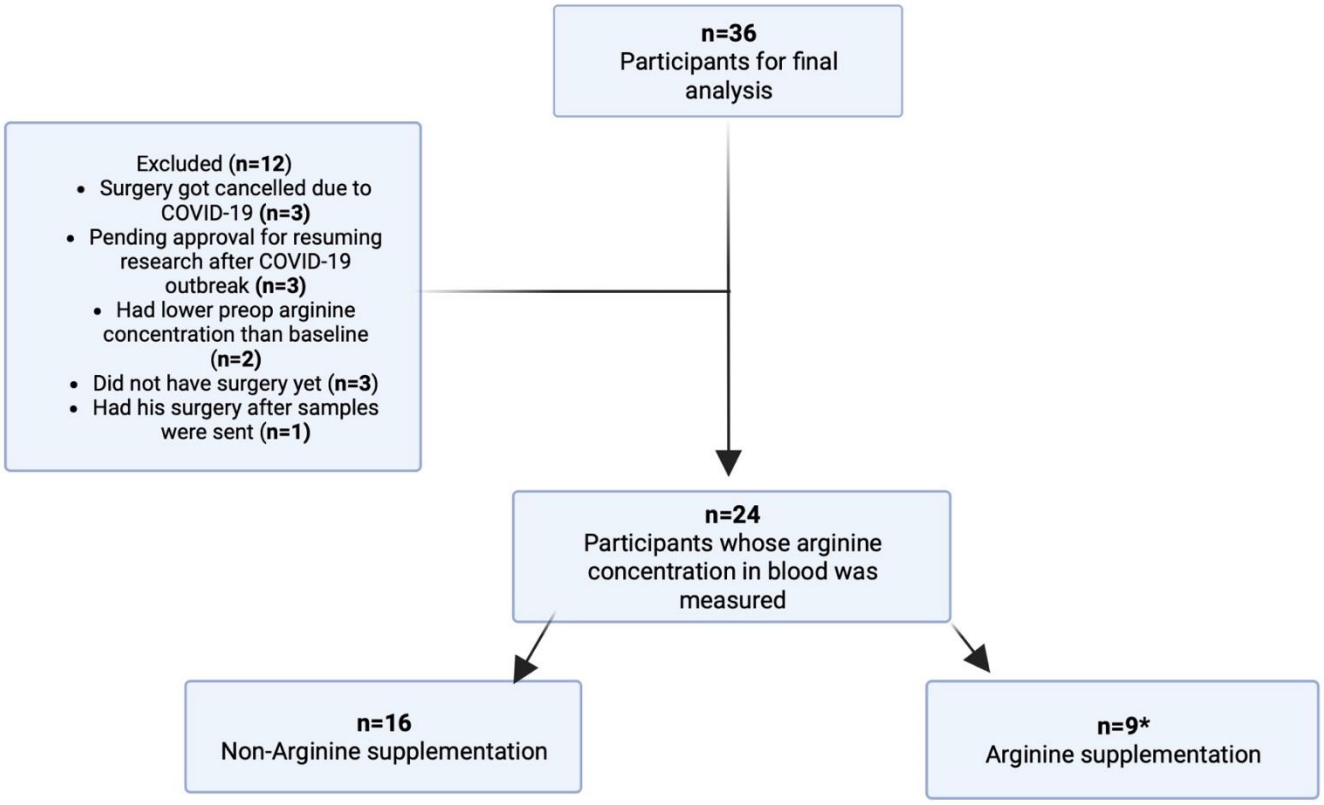


Figure 4: Patients who had their blood samples sent out to measure their arginine concentration. *CT002(RK) and (LK) were both included for analysis and counted in as extra participants.

After RNA extraction from the tibial cartilage tissue of the 36 individuals, n=18 (n=10 non-arginine supplementation and n=8 arginine supplementation) passed to have good quality RNA after RNA clean up (OD ratio 260/280 > 1.8, concentration > 25ng/ul) (Figure 5).

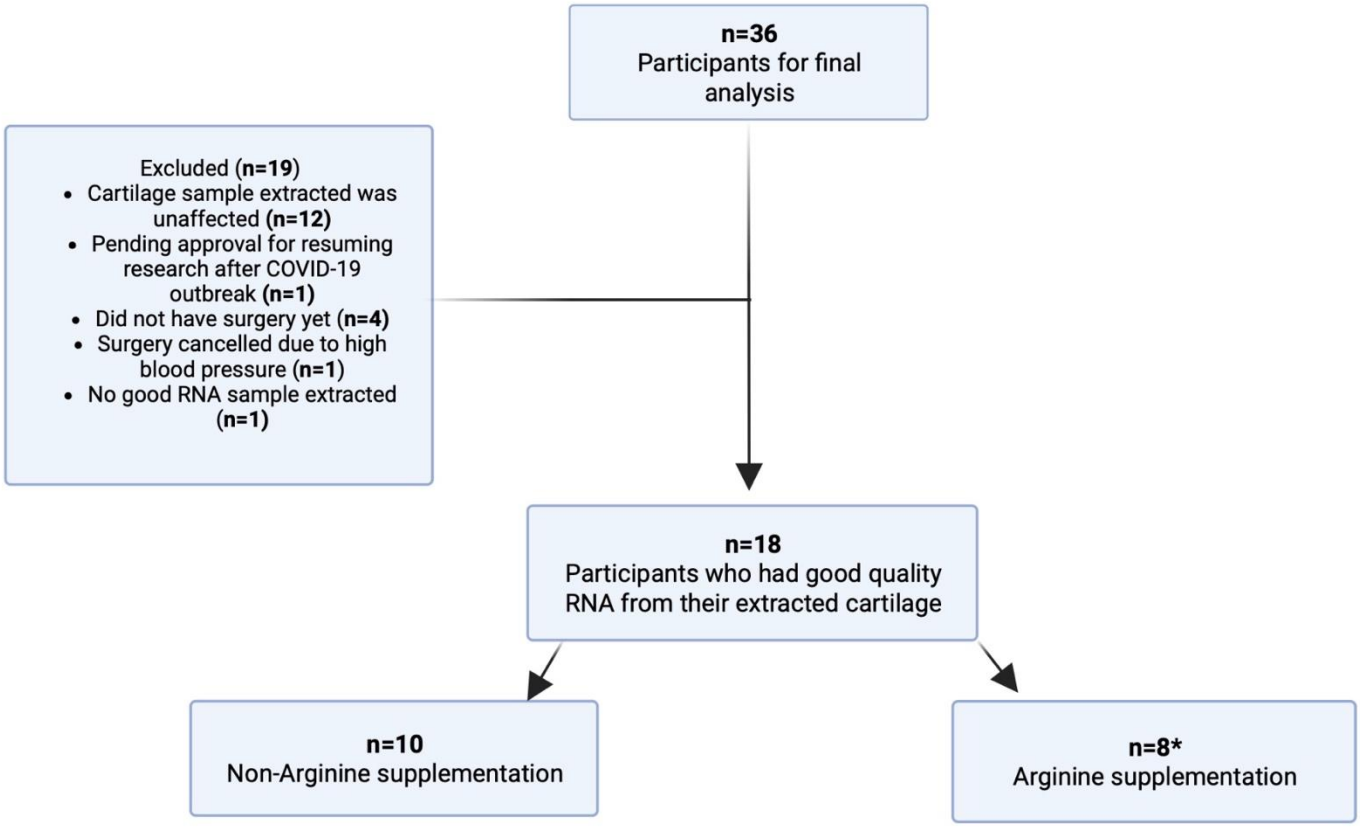


Figure 5: Patients who had good quality RNA from their extracted cartilage sample and had their gene expression level studied. *CT002(RK) and (LK) were both included in gene expression analysis and were counted in as extra participants.

4.6 Randomization and trial procedure

Eligible participants were randomized to receive one of the two treatment arms (1.5g of L-arginine from week 0 to time of TKR or nothing from week 0 to time of TKR) (Figure 2). Treatment allocations were concealed from patients but were blinded to the research assistant and student who collected the joint tissue samples during surgery, performed gene expression assays, and graded OA cartilage tissue. After the baseline clinical visit, all the eligible consenting participants were randomized by computer-generated randomization- the 49 participants were randomized into two groups. One group received a package containing arginine supplements with instructions, and another group, as controls, received nothing but continued their regular care. They were then instructed to take proper dosages per day (3 tablets per day) for six months before their TKR surgery. The supplements were purchased from the market with the correct dosages required for the study within Canada. All the patients had continued their regular health care.

4.7 Arginine administration and management

The arginine supplied to the patients enrolled for this clinical trial was procured from the Local health product retailer – GNC. After the tablets were purchased, they were stored at room temperature in original containers away from light. The containers containing the arginine tablets were handed to patients during the initial interview (after they signed the consent form). Remaining pill count was performed to assess compliance and the returned unused pills were disposed of by submitting the leftover to Medical Laboratory Manager, and she sent them out for proper disposal.

4.8 Demographic and anthropometric data

With the help of a research assistant, demographic information was collected through a self-administered questionnaire. Anthropometric data, including height and weight, were collected from medical records, including hospital admission records. Age was calculated (subtracting the date of surgery – date of birth) at the time of the surgery. Body mass index (BMI) was calculated by dividing weight in kilograms by squared height in meters.

4.9 Specimen collection

4.9.1 Blood collection

Blood samples were collected in three stages of the trial: during the time of taking consent to participate in the study (baseline), right before the surgery(preop), and twelve months after the surgery. During baseline and preop, 4ml of blood was collected using an EDTA tube and later used for plasma separation. The plasma was separated using a standard protocol [105] and stored at −80◦C freezer. The plasma samples were later sent out to The Metabolomics Innovation Centre (TMIC) for metabolomic analyses. They measured the arginine concentration by liquid chromatography–mass spectrometry (LC-MS) method using the Biocrates MxP Quant 500 kit. Another 2.5 ml of blood was collected using a Paxgene Blood RNA tube and stored at -80C freezer. This blood sample was later used for RNA extraction (PAXgene Blood RNA Kit IVD, Qiagen), and the RNA sample was used for RNA-Seq later (Illumina NovaSeq at Genome Quebec). After

12 months post-op, only 4ml blood was collected using an EDTA tube which was used for metabolomics profiling to compare arginine level with previous time points.

4.9.2 Cartilage collection

During the patient's total knee replacement surgery, after joint fragments were removed before hardware fixation, four pieces (about 200mg each) of full-thickness articular cartilage samples were harvested from tibial and femoral osteoarthritic lesions. All the cartilage tissues were then flash-frozen in liquid nitrogen (LN_2) and stored at -80˚C for further use.

4.10 RNA extraction from Human Cartilage Tissue

Cartilage IDs were identified, and DNA and RNA IDs were created in the arginine clinical trial database. The cartilage samples were retrieved from the ultra-low temperature freezer (-80˚C) and checked to ensure no bone was attached. The pieces were then quickly weighed on an analytical balance (TX323L, Shimadzu, Kyoto, Japan), and weight was recorded. Caution was taken when handling the samples, and the samples were then kept in LN_2 right after during the entire process to make sure the samples were not thawed. Prior to RNA extraction, LN_2 was added to the Freezer/Mill to cool the tub. Guanidine thiocyanate (GTC) was warmed at 40-50˚C in a water bath to avoid crystallization. RNA and DNA were extracted from cartilage samples using a previously developed optimal RNA/DNA extraction protocol [135][137]. 1ml of TRIzol Reagent (Invitrogen, Waltham, United States) and 150 ul of 6M guanidine thiocyanate (Sigma-Aldrich, St. Louis, United States) were added to a homogenizing cylinder carefully inside

a fume hood. The cylinder was then placed in the Freezer/Mill tub (6770, Spex SamplePrep, Metuchen, United States) to freeze the solution.

Once the solution was frozen, up to 200mg of the frozen cartilage sample was transferred to the cylinder, followed by an impactor (to help break the cartilage sample). The cylinder was then sealed with an end plug and inserted into the grinding chamber of the Freezer/Mill. Individual cartilage samples were powdered using the following parameters: pre-cooling of the samples for 10 minutes; 3 cycles of 1 minute each at the maximum frequency with a cooling time of 3 minutes between grinding cycles. The homogenized sample was then carefully transferred to a 50 ml tube to thaw at room temperature (RT). After the sample had thawed and reached RT, it was incubated at RT for another 5 minutes and then transferred to a 2ml RNase-free tube, and 0.25 ml of chloroform (Fisher, Waltham, United States) was added and mixed well using a pipette. This mixture was then shaken vigorously by hand for 15 seconds and incubated at RT for 2-3 minutes. The mixture in the 2ml tube was then centrifuged at 12,000 x g for 15 minutes at 4°C. After centrifugation, the aqueous phase was carefully transferred to a new 2ml tube (without taking any interphase) for RNA extraction. An equal volume of 70% ethanol was added to the aqueous phase and mixed with a pipette, and the mixture was then loaded onto a RNeasy spin column (Qiagen, Hilden, Germany). RNA was extracted using the RNeasy mini kit following the manufacturer's protocol. The remaining organic phase was kept at 4 ˚C for DNA extraction. Absorbance ratios (OD_{260}/OD_{280} and OD_{260}/OD_{230}) and concentration of RNA samples were measured using NanoDrop 1000 spectrophotometer

(ThermoFisher, Waltham, United States) and recorded in the arginine clinical trial database.

4.11 DNA extraction from Human Cartilage Tissue

Before DNA extraction, Buffer ATL was warmed at 56°C to dissolve precipitation and mixed if necessary. A previously developed protocol was used for DNA extraction[135] [137]. First, 0.3 ml of 100% ethanol was added to the organic phase per 1 ml of Trizol reagent that was used for sample grinding. The tube was then vortexed to mix the organic phase with ethanol thoroughly. After incubation at RT for 2-3 minutes, the sample was centrifuged at 8,000 x g for 5 minutes at 4°C. The supernatant was discarded, and another 0.3 ml of 100% ethanol was added. The sample was vortexed to break up the pellet and centrifuged again at 8,000 xg for 5 minutes at 4°C. The supernatant was discarded, and then 180 ul Buffer ATL and 20 ul proteinase K were added per 35-40 mg cartilage tissue, and the tube was vortexed again. The mixture was then incubated in the thermo shaker (MaxQ™ 4000, ThermoFisher, Waltham, United States) at 300 rpm at 56°C overnight. If digestion was incomplete after 24 hours, an extra 10% of the original volumes of ATL and proteinase K were added, and digestion was extended for a few hours. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (Invitrogen, Waltham, United States) was then added. The sample was shaken vigorously for 20 seconds and then centrifuged at 16,000 xg for 5 minutes at RT. The supernatant was then carefully transferred to a new 2 ml tube, and an equal volume of chloroform: isoamyl alcohol (24:1) (Sigma, St. Louis, United States) was added. The sample was shaken thoroughly for 20 seconds and centrifuged at 16,000 xg for 5 minutes at RT. The supernatant was carefully transferred to

a new 2 ml tube, and 2~3× volume of 100% ethanol was added. The tube was inverted a few times to mix the solution and then incubated at -80°C for >30 minutes or -20°C overnight to precipitate DNA. The sample was then centrifuged at 20,000 x g for 30 minutes at RT, and the supernatant was discarded. The pellet was washed with 70% ethanol and further centrifuged at 20,000 x g for 15 minutes. This step was repeated another 3 times (4 times in total). The pellet (DNA) was left to air dry, and then 50 ul TE buffer was added to dissolve the DNA. Absorbance ratios (OD_{260}/OD_{280} and OD_{260}/OD_{230}) and concentration were then measured using NanoDrop 1000 spectrophotometer. The DNA samples extracted during the study were not used for analysis for this study and were stored in an ultra-low temperature freezer (-80˚C) for future use.

4.12 Gene expression measurement

4.12.1 Qubit measurement

After the extracted RNA samples were measured by NanoDrop spectrophotometer, the samples were measured with Qubit RNA HS Assay Kits (Invitrogen, Waltham, United States). Two assay tubes were set up for standards (standard 1 and standard 2) and one tube for each sample. 200 ul Qubit working solution was prepared for each standard and sample by diluting the Qubit RNA HS Reagent 1:200 in Qubit RNA HS Buffer. 0.5 mL Qubit assay tubes (Invitrogen, Waltham, United States) were labeled, and standards and samples were prepared (Table 7).

Table 7: Steps of preparing standards and samples for measurement of RNA sample using Qubit RNA HS Assay Kit.

Volume	Standards	Samples
Working solution	190 uL	199 uL
Standard (1 and 2)	10 uL	-
Sample	-	1 uL
Total in each assay tube	200 uL	200 uL

The standards and samples were then vortexed for 2-3 seconds and incubated at RT for 2-3 minutes. A standard curve was generated using the two standards. The RNA samples were then measured on the Qubit 2.0 Fluorometer (Invitrogen, Waltham, United States). The stock concentrations of the samples were calculated as Qubit concentration X 200 and recorded in the arginine clinical trial database.

4.12.2 cDNA synthesis

After measurement of the extracted RNA using Qubit, complimentary DNA (cDNA) was synthesized using the cDNA Synthesis Kit: SuperScript™ IV VILO™ (SSIV VILO) Master Mix with DNase (Invitrogen, Waltham, United States) following manufacturer's manual. 200 ng of RNA was used for each sample for cDNA synthesis. The volume of RNA used was calculated as 200ng/Qubit stock concentration (ng/ul). Prior to cDNA synthesis, two programs were created in the thermal cycler (MasterCycler Gradient, Eppendorf, Hamburg, Germany): "EZDNASE" for DNase digestion and "CDNAVILO" for cDNA synthesis. The lid of the thermal cycler was preheated to 104˚C. The samples were then prepared in 8-tube strips on ice, each containing ezDNase buffer, ezDNase, RNA, and nuclease-free water in varying volumes. One no-template control (NTC) was

used as control using H_2O as a template instead of RNA. Sample tube strips were vortexed and centrifuged briefly and then inserted into the thermal cycling block, and the program "EZDNASE' was run.

Meanwhile, the cDNA synthesis master mix containing SuperScript™ IV VILO master mix (4 ul for each sample) and nuclease-free water (6 ul for each sample) was prepared on ice. Once DNase treatment was completed, samples were taken out of the thermal cycler immediately and centrifuged briefly, and then kept on ice. 10 ul of the cDNA synthesis master mix was added into each tube, and the tube strips were then capped with new cap strips. The tube strip was vortexed, centrifuged briefly, and then inserted into the thermal cycling block, and the program "CDNAVILO" was run. Once the cDNA synthesis reaction was completed, samples were taken out of the thermal cycler and centrifuged briefly. cDNA samples and NTC were transferred to 1.5 ml tubes on ice. Each sample was aliquoted into two separate tubes and stored at -80°C.

4.12.3 Quantitative PCR

Relative expression levels of cartilage degradation enzymes - *MMP13, CTSK, CTSB*, and cartilage syntheses genes - *ACAN*, *COL2A1* were analyzed by quantitative polymerase chain reaction (qPCR) using an ABI ViiA Real-Time PCR system. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal control gene to normalize cDNA input for each sample. The relative quantification (RQ) of each gene in each sample was calculated as fold-changes using the Livak method (delta-delta Ct method).

Prior to the qPCR experiment, a run file was created using QuantStudio software (Applied Biosystems, Waltham, United States). *MMP13, CTSK, CTSB, ACAN, and COL2A1* were set as target genes, and *GAPDH* was set as the endogenous control. The reporter was set as "SYBR," and the "Quencher" set as none. Different colors were selected to visualize each gene. "Passive reference" was set as ROX. Sample names were defined, and samples and genes were assigned to the 96-plate wells (PCR MicroPlate PCR-96-FLT-C, Axygen, ThermoFisher). NTC was used for each gene to detect contamination in the reaction. Each sample/NTC and gene combination was set to run in triplicates.

The cDNA samples and Power SYBR® Green PCR Master Mix (Applied Biosystems, Waltham, United States) were first taken out of the -20˚C freezer and thawed on ice. Next, the qPCR master mix was prepared using Power SYBR® Green PCR Master Mix and individual primer sets for genes *GAPDH, MMP13, CTSK, CTSB, ACAN*, and *COL2A1* (Table 8). The sequences of primers are presented in Table 9.

Table 8: The qPCR master mix preparation

Component	Vol 1x (ul)	*GAPDH/MMP3/CTSB/CTSK/ACAN/ COL2A1* (number of wells containing cDNA sample and NTC) x (ul)
Power SYBR Green Master Mix (2X)	10	_ x 10
Forward primer (10uM)	0.4	_ x 0.4

Reverse primer (10uM)	0.4	_ x 0.4
Nuclease free water	4.2	_ x 4.2
Total	15	

Table 9: Primer sequences for qPCR

Gene	Forward primer	Primer sequence (5'-3')	Reverse primer	Primer sequence (5'-3')	Amplicon size (dp)
GAPDH	GAPDH-Q-L	GCAAATTCCATGGCACCGT	GAPDH-Q-R	TCGCCCCACTTGATTTTGG	106
MMP13	MMP13-Q92L	AGCTGGACTCATTGTCGGGC	MMP13-Q92R	AGGGTAGCGCTCTGCAAACTGG	92
CTSB	CTSB-Q77F	CCAAGCCACCCCAGAAGAGTT	CTSB-Q77R	TGGCCATTGTTCCCGTGCAT	77
CSTK	CTSK-Q52F	TTCCATCAGCAGGATGTGGGG	CTSK-Q52R	GCTCACCACAGGTAGCAGCAG	52
ACAN	ACAN-Q62F	GTCGTGGTGAAAGGCATCGT	ACAN-Q62R	AAGTCGAGGGTGTAGCGTGT	62
COL2A1	CTXII-Q84F	CTCTGGTGCCGAAGGTCCAC	CTXII-Q84R	CTCTCACCACGTTGCCCAGG	84

Each cDNA sample was 1:80 diluted using nuclease-free water, and 5ul of diluted cDNA was added to each well of the 96-well plate. QPCR master mix was briefly vortexed, and 15ul of the master mix was added to each well. The 'reaction volume per well" was set to 10ul and the run (Table 10).

Table 10: PCR condition for amplification

Hold stage		Repeat 40		Melt curve (Continuous)		
50°C	95°C	95°C	60°C	95°C	60°C	95°C
2:00	10:00	0:15	1:00	0:15	1:00	0:15

The plate was vortexed on a shaker (MS 3 digital, IKA, Staufen, Germany) and then centrifuged briefly to break down any bubbles present. The plate was then loaded onto Applied Biosystems ViiA 7 Real-Time PCR System (Applied Biosystems, Waltham, United States), and the previously created file was run. Once the qPCR run was completed, the file was saved, and results were read using QuantStudio. The amplification curves and melt curves were inspected. The baseline and threshold were set up manually where necessary. The results were then exported to excel files for further data analysis using the following steps:

a) Calculate Ct mean: Ct mean of the triplicates
b) Calculate delta Ct: (Ct mean of target gene)- (Ct mean of GAPDH of that sample)
c) Calculate delta delta Ct: One control sample selected (cDNA8) as a calibrator, (delta Ct of other samples) – (delta Ct of calibrator)
d) Calculate RQ (relative quantity) value: POWER(2,-delta delta Ct)

4.12.4 Amplicon confirmation by 2% agarose gel electrophoresis

After the qPCR run, randomly selected PCR products from the sample plate were used for amplicon confirmation using gel electrophoresis. The concentration of the agarose gel was set to 2% (w/v) based on the sizes of the PCR products. For the preparation of the agarose gel, 2 g of ultra-pure agarose (Invitrogen, Waltham, United States) was weighed using an analytical balance and combined with 100 ml of 1 x Buffer TBE (89 mM Tris-borate, 2 mM EDTA) in a flask. The agarose/buffer mixture was heated using a microwave until it came to a boil, and the agarose was completely dissolved. The agarose gel was then allowed to cool to ~60˚C on a benchtop. Then 10 ul of SYBR™ Safe DNA Gel Stain (Invitrogen, Waltham, United States) was added and mixed with the gel. The mini gel tray (Bio-Rad, Hercules, United States) was placed into the mini gel caster (Bio-Rad, Hercules, United States) or taped at the open edges to create a mold. A 1.5mm 15-well comb (Bio-Rad, Hercules, United States) was placed into the gel tray to create the wells. Then the molten agarose gel was poured into the gel mold. Bubbles in the gel were removed, and the gel was allowed to be set at 4˚C and protected from light. The comb was then carefully removed, and the gel was placed in the gel cell (Mini-Sub Cell GT Cell, Bio-Rad, Hercules, United States). Running buffer 1 x Buffer TBE was poured into the cell to cover the surface of the gel. 4 ul of TriTrack DNA Loading Dye (6X, Thermo Scientific, Waltham, United States) was added to each of the selected wells of the 96-well plate and mixed with the PCR products using a pipette. 15 ul of the sample/dye mixture was loaded into each well, and 10 ul of Low range DNA ladder (Thermofisher, Catalog number SM1191, SM1192, Massachusetts, United States) was loaded into the wells on

both sides. The electrodes of the gel cell were connected to the power supply (PAC3000, Bio-Rad, Hercules, United States), and the gel was run at a voltage of 100 V for 45 minutes or until the first band of the dye reached 2/3 of the gel length. When electrophoresis was completed, the power supply was turned off, and the bands were visualized under UV using the U: GENIUS Gel Imaging System (Syngene, Bengaluru, India). Gel images were taken and saved.

4.13 Statistical analyses

All statistical analyses were performed using R. Appropriate parametric tests like T-test and paired t-test and non- parametric tests like Chi square and Mann Whitney tests were used to compare differences between treatment and control groups for gene expressions. Statistical significance was at alpha level = 0.05.

The orthotoolkit, an online score developer, was utilized to input individual scores obtained from the SF-36 questionnaires. This tool generated a summarized report, from which the relevant data was extracted and subsequently subjected to further analysis [138]. This tool has been validated and used in other population studies [142].

5 RESULTS

5.1 Descriptive analysis

5.1.1 Demographic information

The purpose of this clinical trial was to evaluate the effect of oral L-arginine supplementation on knee OA in a sample of patients scheduled to undergo total knee

replacement surgery due to primary knee OA. The clinical trial involved a diverse sample of participants aged 40 years and older, with a roughly equal gender distribution.

A total of 380 individuals who met the inclusion criteria were approached via phone calls by the research assistant. Out of the 380 individuals, 49 participants agreed to participate in the trial, resulting in a low response rate of 13%. The reasons for the low participation rate may vary from lack of interest or time constraints to concerns over side effects of the supplementation to health and concerns over privacy and confidentiality. Despite the low response rate, the clinical trial still managed to collect valuable data from the willing participants, shedding light on the possible effects of arginine supplementation on OA.

Of the 49 individuals, one individual was identified to be taking vitamin C supplements and was excluded from the study. The remaining 48 individuals were randomized equally to take no supplementation (n=24) and arginine supplementation (n=24). From the 24 individuals allocated to take arginine supplementation, one individual was identified by the orthopedic surgeon during their total knee replacement surgery to be suffering from rheumatoid arthritis and was thus excluded from the study. A total of 11 individuals withdrew their consent at different stages of the trial. All of the individuals who had withdrawn their consent were from the group randomized to take arginine supplementation. One individual had no affected tibial sample collected by the orthopedic surgeon during his knee replacement surgery and was excluded from the trial as only affected tibial samples were taken for analysis. This resulted in a total of 36 individuals to be included for descriptive analysis, with 12 individuals taking arginine supplementation and 24 individuals as controls having no supplementation over the course of the trial (Figure. 6)

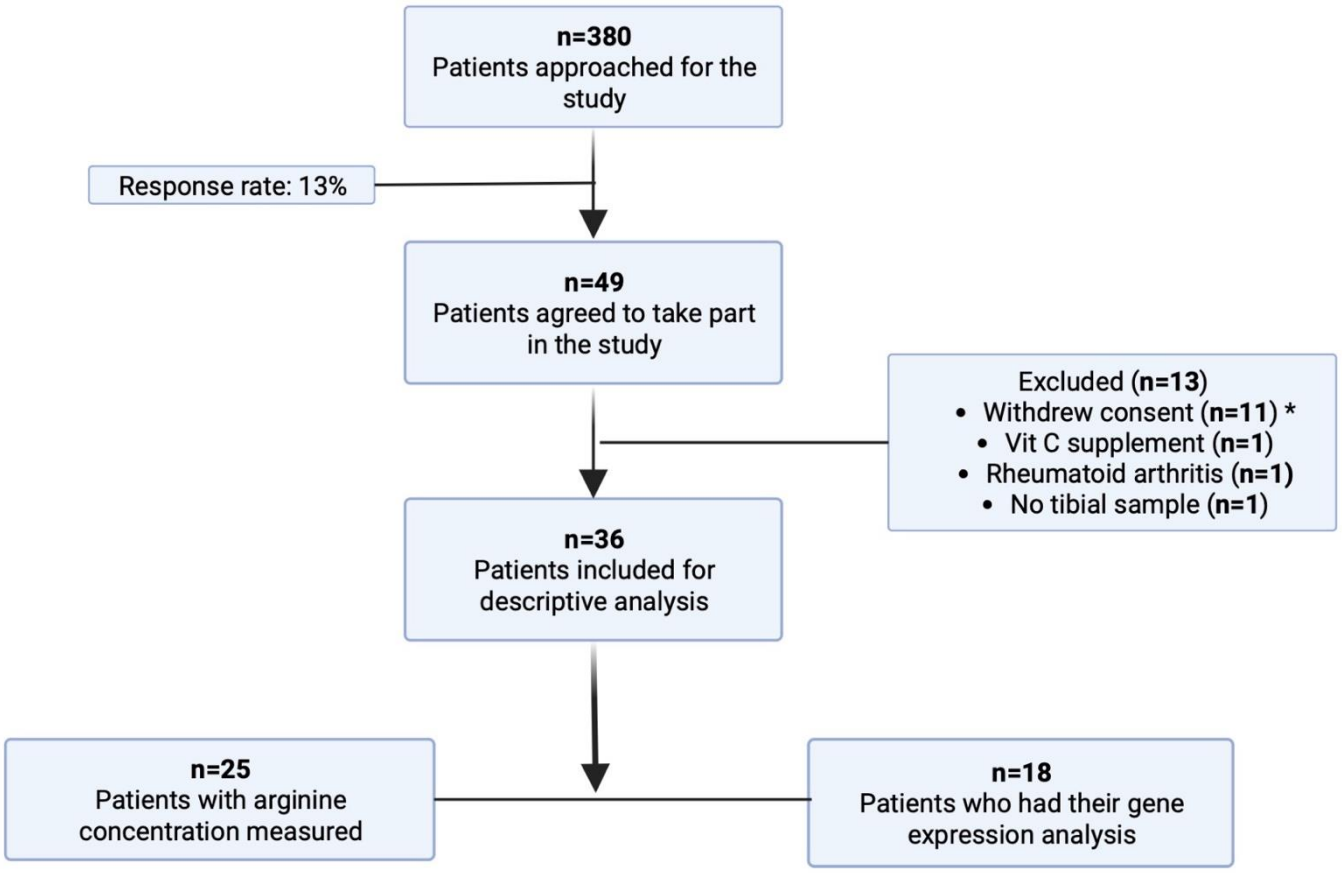


Figure 6: Patient disposition chart.

Of the 36 participants, 25 individuals (CT002 was counted as two individuals as he had surgery on both his right and left knee) had their arginine concentration levels measured. Twelve individuals were excluded for various reasons (Table 11), such as lower preop concentration than baseline level, or no available data that could affect the accuracy of the results.

Table 11: Participants who had their arginine concentration measured with the reason for exclusion.

ARGININE		
STUDY ID	Included/Excluded	Reason for exclusion
CT002(RK)	Included	
CT002(LK)	Included	
CT005	Included	
CT008	Included	
CT009	Included	
CT010	Included	
CT013	Excluded	Lower preop than baseline
CT019	Excluded	He had his surgery late
CT025	Included	
CT026	Included	
CT034	Included	
CT040	Excluded	Did not have his surgery yet
CT048	Excluded	Lower preop than baseline
CONTROL		
STUDY ID	Included/Excluded	Reason for exclusion
CT003	Included	
CT004	Included	
CT006	Included	
CT007	Included	
CT011	Included	
CT015	Included	
CT018	Included	
CT020	Excluded	Surgery canceled
CT022	Included	
CT023	Included	
CT024	Included	
CT027	Excluded	COVID-19 Pending approval for resuming research
CT030	Included	
CT031	Excluded	COVID-19 Pending approval for resuming research
CT032	Excluded	Did not have his surgery yet
CT033	Included	
CT036	Included	
CT037	Excluded	COVID-19 Pending approval for resuming research
CT038	Included	

CT042	Excluded	Had surgery after blood samples were sent out
CT043	Excluded	Did not have his surgery yet
CT046	Included	
CT047	Excluded	The appointment canceled due to the patient's ill health
CT049	Included	

Of the 36 participants, 17 individuals (CT002 was counted as two individuals as he had surgery on both his right and left knee) had good affected tibial cartilage samples to extract gene expression data. 19 individuals were excluded due to various reasons (Table 12), such as the cartilage samples extracted were all from the unaffected sites or they did not have their surgeries yet, which could affect the accuracy of the results. One individual CT005 later showed very high gene expression RQ values and was excluded from the analysis because it was an outlier. This resulted in a total of 17 individuals with good gene expression data.

Table 12: Participants who had good, affected cartilage samples for gene expression analysis with the reason for exclusion.

ARGININE		
STUDY ID	Included/Excluded	Reason for exclusion
CT002(RK)	Included	
CT002(LK)	Included	
CT005	Excluded	High gene expression RQ values
CT008	Excluded	Cartilage samples extracted were all from unaffected site
CT009	Included	
CT010	Excluded	Cartilage samples extracted were all from unaffected site
CT013	Included	
CT019	Included	

CT025	Excluded	Cartilage samples extracted were all from unaffected site
CT026	Included	
CT034	Excluded	Cartilage samples extracted were all from unaffected site
CT040	Excluded	Did not have his surgery yet
CT048	Included	
CONTROL		
STUDY ID	Included/Excluded	Reason for exclusion
CT003	Excluded	Cartilage samples extracted were all from unaffected site
CT004	Excluded	Cartilage samples extracted were all from unaffected site
CT006	Excluded	Cartilage samples extracted were all from unaffected site
CT007	Excluded	Cartilage samples extracted were all from unaffected site
CT011	Excluded	Cartilage samples extracted were all from unaffected site
CT015	Included	
CT018	Included	
CT020	Excluded	Pending approval for resuming research after COVID-19 outbreak
CT022	Excluded	No good RNA sample was extracted from this individuals samples
CT023	Included	
CT024	Included	
CT027	Included	
CT030	Excluded	Cartilage samples extracted were all from unaffected site
CT031	Excluded	Did not have his surgery yet

CT032	Excluded	Did not have his surgery yet
CT033	Excluded	Cartilage samples extracted were all from unaffected site
CT036	Included	
CT037	Included	
CT038	Excluded	Cartilage samples extracted were all from unaffected site
CT042	Included	
CT043	Excluded	Did not have his surgery yet
CT046	Included	
CT047	Excluded	The appointment canceled due to the patient's ill health
CT049	Included	

5.1.2 Age

From the total of 49 study participants, age was recorded at two different time intervals, one at recruitment (baseline) and one during the preop blood collection date. The sample included both males and females, with a mean age of 66.6 ± 9.1 years at baseline and 67.7 ± 9.2 years at the preop blood collection date. The age range of the participants was 40 to 80 years. The mean age was 67.5 ± 9.2 at baseline and 68.5 ± 9.3 at the preop blood collection date for those who took arginine supplementation and 66.2 ± 9.1 at baseline and 67.3 ± 9.4 at preop blood collection date for controls. The age difference between the two groups (treatment and control) at baseline was not significant at $p = 0.68$. The Mann-Whitney U test was used in the analysis. The age difference between the sexes between the two groups is shown in Table 13.

Table 13: The age difference between sexes between participants having arginine supplementation and no-supplementation.

Arginine			Control		
Variable	Female	Male	Variable	Female	Male
Age at baseline (mean +/- SD)	64.2 ± 9.9	69.8 ± 8.7	Age (baseline)	61.4 ± 9.9	69.6 ± 6.8
Age at preop (mean +/- SD)	65.2 ± 10.9	70.3 ± 8.6	Age (pre-op)	62.2 ± 10.1	71.5 ± 6.4

5.1.3 Sex

The clinical trial aimed to explore the effect of arginine supplementation on OA in a sample of participants. The study comprised of 36 participants, consisting of 15 females and 21 males. Among those who received arginine supplementation, 42% were female, and 58% were male. Similarly, in the control group that did not receive supplementation, 42% were female, and 58% were male. A comparison of the gender distribution between cases and controls yielded non-significant results with p=1, indicating no statistically significant gender difference between the groups (Table 14). The chi-squared test was used for analysis.

Table 14: Gender distribution between cases and control.

Arginine			Control		
Variable	Female	Male	Variable	Female	Male
Sex (%F)	42%(5/12)	58%(7/12)	Sex (%F)	42%(10/24)	58%(14/24)

5.1.4 Body mass index (BMI)

From the 36 individuals who had their descriptive data, BMI was calculated in both males and females. Individual height and weight were recorded at the time of recruitment, and BMI was calculated by dividing weight in kilograms by the square of height in meters (kg/m^2). The mean BMI was 35.1 ± 8.9 for the participants who received arginine supplementation and 34.2 ± 11.9 for the control group. There was no statistically significant difference in BMI between the two groups (p=0.34), as determined using the Mann-Whitney U test. A detailed breakdown of the differences in BMI between males and females in each group is presented in Table 15.

Table 15: BMI distribution between sexes in cases and control.

Arginine			Control		
Variable	Female	Male	Variable	Female	Male
BMI (kg/m^2) ± SD	40.9 ± 11.1	30.8 ± 3.8	BMI (kg/m^2) ± SD	41.1 ± 14.8	29.3 ± 6.3

5.2 Analysis of primary outcome

5.2.1 Arginine concentration

In this study, metabolomics profiling was performed with using a commercially available metabolomic assay kit MxP Quant 500 at The Metabolomics Innovation Centre (TMIC), which included arginine concentration levels in blood samples collected from participants at two-time points: baseline and preoperative. Paired samples were analyzed to determine the percentage change between these two-time points in both groups. The percentage change was calculated using the formula ((preop-baseline)/baseline) multiplied by 100

and then averaged across the individual study IDs within each group. The baseline arginine concentration was found to be 66.9 ± 16.3 μMol for participants receiving arginine supplementation and 69.1 ± 20.30 μMol for those who did not receive supplementation. At the time of surgery, the arginine concentration was 81.6 ± 20.43 μMol for the supplementation group and 84.9 ± 20.70 μMol for the control group. Both groups exhibited a lower baseline arginine concentration that increased by 23.5% ± 22.6 and 33.5% ± 59.2 in the supplementation and control groups, respectively. However, there was no statistically significant difference in the percentage change between the two groups (p=0.76), as determined using the Wilcoxon signed-rank test (Table 16).

Table 16: Arginine concentration in arginine supplementation group and controls and the percentage change between two-time points.

Variable	Arginine supplementation (n=9)	Non-arginine supplementation (n=16)	P value
Arginine concentration at baseline (μMol)	66.9 ± 16.3	69.1 ± 20.30	0.77
Arginine concentration at surgery (μMol)	81.6 ± 20.43	84.9 ±20.70	0.71
Arginine concentration percentage change between baseline and surgery	23.5 ± 22.6	33.5 ± 59.2	0.76

5.2.2 Gene expression analysis of *MMP13, CTSK, CTSB, ACAN & CTXII*

In this study, the expression levels of five target genes: *MMP13, CTSB, CTSK, ACAN,* and *COL2A1*, were compared between an arginine supplementation group (n=7) and a control group (n=10). The data showed that *MMP13* expression was higher in the arginine group compared to the control group, with mean values of 5.21 ± 7.18 and 2.57 ± 4.23, respectively, although the difference was not statistically significant (p=0.31). The expression levels of *CTSB* and *CTSK* did not show a significant difference between the two groups, with mean values of 1.08 ± 0.57 and 0.92 ± 0.18 (p=0.96) for *CTSB* and 1.19 ± 0.57 and 1.05 ± 0.24 (p=0.89) for *CTSK* in the arginine and control groups, respectively. Similarly, there was no significant difference in *ACAN* expression levels between the arginine and control groups (0.88 ± 0.60 and 1.01 ± 0.26, respectively, p=0.23). The expression of *COL2A1* showed a trend towards being lower in the arginine group compared to the control group, with mean values of 0.80 ± 0.28 and 1.39 ± 0.50, respectively, although the difference was not statistically significant (p=0.19). The Mann-Whitney U test was used in the analysis of these five genes. These findings suggest that arginine supplementation may have a moderate effect on *MMP13* expression but does not significantly affect the expression of *CTSB, CTSK, ACAN,* or *COL2A1* (Table 17).

Table 17: RQ value of cartilage degradation-related genes from RT-PCR

Target gene	Arginine (n=7)	Control (n=10)	P value
MMP13	5.21 ± 7.18	2.57 ± 4.23	0.31
CTSB	1.08 ± 0.57	0.92 ± 0.18	0.96
CTSK	1.19 ± 0.57	1.05 ± 0.24	0.89
ACAN	0.88 ± 0.60	1.01 ± 0.26	0.23
COL2A1	0.80 ± 0.28	1.39 ± 0.50	0.19

5.3 Analysis of secondary outcome

5.3.1 WOMAC score

The WOMAC score is a widely used measure of pain, stiffness, and physical function in patients with knee osteoarthritis. The WOMAC scores were obtained from all participants at four different timelines: at the time of recruitment (baseline), preop, at 6 months and 12 months after the total knee replacement surgery. In this study, 12 patients were treated with arginine supplementation and compared with a control group of 24 patients who got no supplementation (control group).

Table 18 shows that there is no statistical difference in WOMAC scores at baseline between the two groups.

Table 18: WOMAC scores between two groups at baseline.

Variables	Arginine supplementation (n=12)	Non-arginine supplementation (n=24)	P-value
WOMAC pain at baseline	12.2 ± 4.8	12.8 ± 3.1	0.81
WOMAC stiffness at baseline	5.3 ± 1.5	5.9 ± 1.3	0.24
WOMAC function at baseline	37.3 ± 15.8	43.9 ± 9.7	0.17
WOMAC total score at baseline	54.8 ± 21.0	62.6 ± 12.4	0.29

Further results showed that the group receiving arginine supplementation exhibited significant improvements in all WOMAC measures at 6 and 12 months compared to their baseline scores. This indicated a notable enhancement in knee pain, stiffness, and function. Specifically, the WOMAC pain score decreased by 68.9% at 6 months and 77%

at 12 months from baseline. The WOMAC stiffness score decreased by 62.3% at 6 months and 64.1% at 12 months from baseline. The WOMAC function score decreased by 60.9% at 6 months and 61.1% at 12 months from baseline. Additionally, the WOMAC total score decreased by 62.8% at 6 months and 64.9% at 12 months from baseline (Figure 7-10). Notably, although not statistically significant, the pain score at 12 months was slightly better in the arginine group compared to the control group.

Similarly, the control group also exhibited significant improvements in their WOMAC scores at 6 and 12 months compared to baseline. This suggests that the substantial improvements observed at 6 and 12 months may be attributed primarily to the effectiveness of the replacement surgery. However, further research is necessary to confirm the effect of arginine on WOMAC scores.

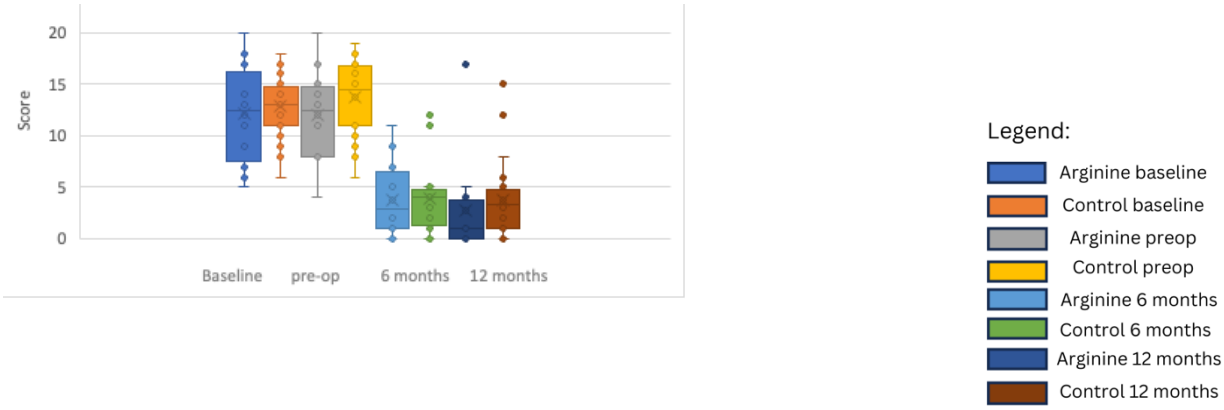


Figure 7: WOMAC scores for pain

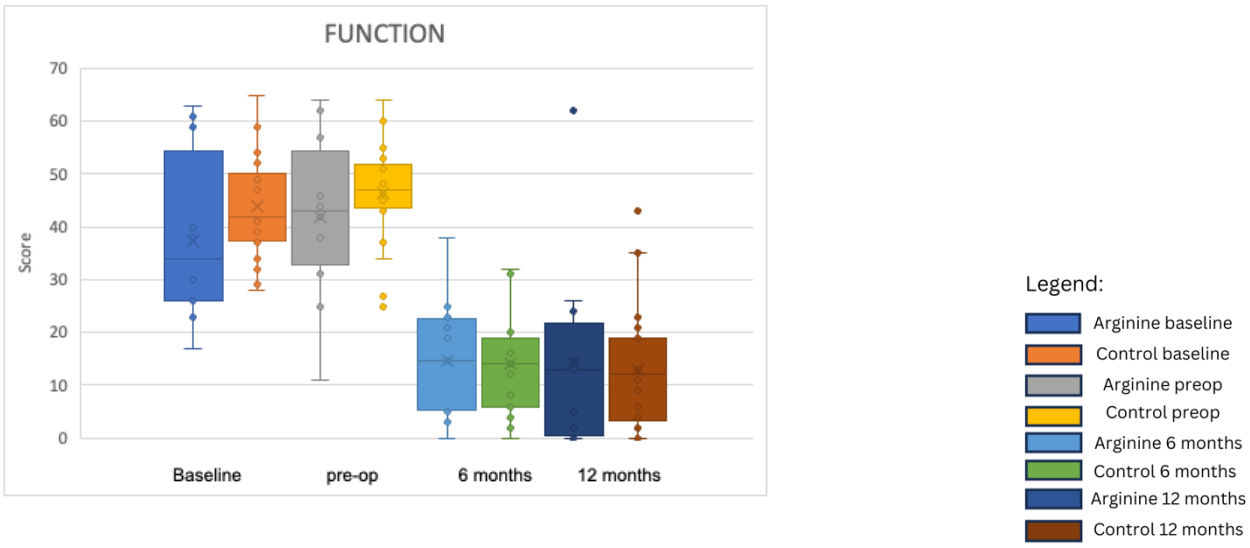


Figure 8: WOMAC scores for function

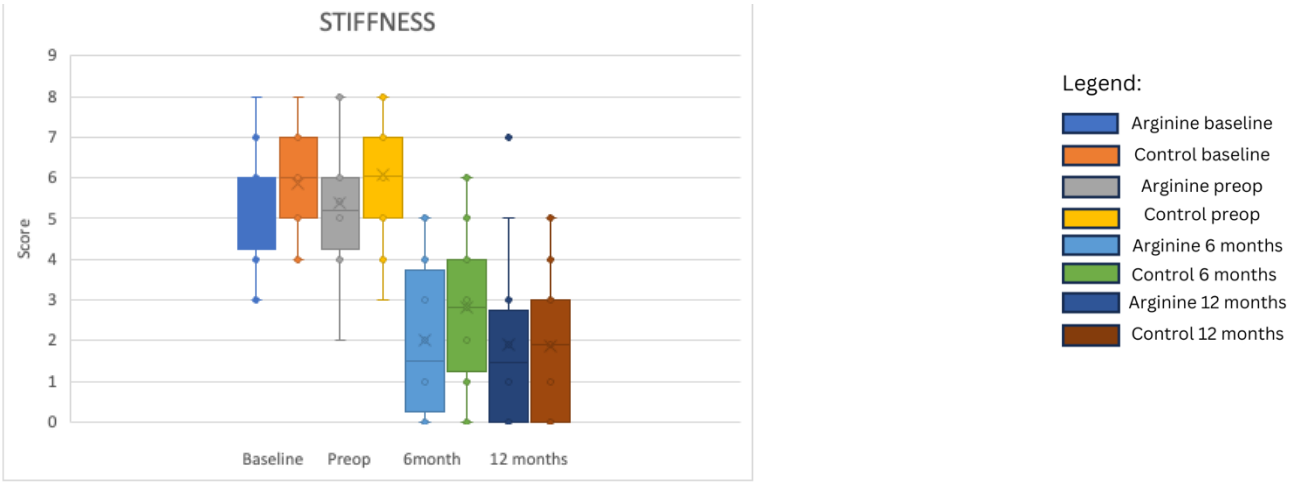


Figure 9: WOMAC scores for stiffness

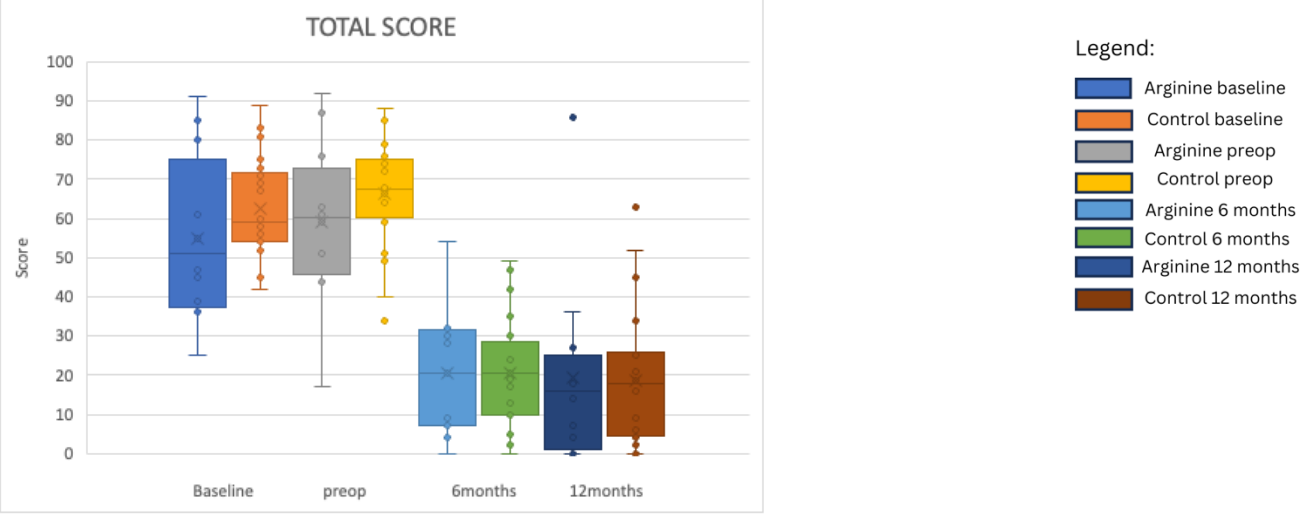


Figure 10: WOMAC total score

5.3.2 SF-36 score

The study compared the effects of arginine supplementation versus non-arginine supplementation on various aspects of health-related quality of life using the SF-36 questionnaire. The arginine group (n=12) and control group (n=24) were assessed at baseline, pre-op, 6 months, and 12 months. At baseline, there were no statistically significant difference between the two groups on any of the eight dimensions of the SF-36 questionnaire, except for Physical Functioning (PF). Specifically, the arginine supplementation group had a higher mean (indicating better health status) score of 27.5 ± 11.8 than non-arginine group 21.25 ± 17.8, but this difference was not large enough to reach statistical significance (p-value 0.10) (Table 19).

Table 19: SF-36 scores between two groups at baseline.

Variables	Arginine supplementation (n=12)	Non-arginine supplementation (n=24)	P-value
SF-36 Physical Functioning at baseline: (PF)	27.5 ± 11.8	21.25 ± 17.8	0.10
SF-36 Role limitations due to physical health at baseline: (RP)	22.9 ± 39.1	16.6 ± 34.3	0.61
SF-36 Pain at baseline: (BP)	39.0 ± 26.4	33.6 ± 15.6	0.47
SF-36 General Health at baseline: (GH)	57.2 ± 26.2	57.6 ± 20.6	0.89
SF-36 Energy/fatigue at baseline: (VIT)	40.1 ± 27.9	39.6 ± 29.1	0.39
SF-36 Social Functioning at baseline: (SF)	66.6 ± 32.1	58.3 ± 26.7	0.34
SF-36 Role limitations due to emotional problems at baseline: (RE)	75.0 ± 45.2	68.1 ± 44.4	0.60
SF-36 Emotional well-being at baseline: (MH)	76.3 ± 18.9	75.3 ± 15.6	0.72

Further results showed that the arginine-supplementation group had significantly higher scores on all SF-36 measures at 6 and 12 months compared to baseline, indicating an improvement in physical functioning, role limitations to physical health, pain, general health, energy/fatigue, social functioning, role limitations due to emotional problems and emotional well-being. Specifically, the SF-36 pain (BP) score increased from 39.0 ± 26.4 at baseline to 70.8 ± 19.9 at 6 months. The SF-36 physical functioning (PF) score increased from 27.5 ± 11.8 at baseline to 61.0 ± 19.9 at 6 months and 46.0 ± 27.4 at 12 months. The general health (GH) scores increased from 57.2 ± 26.2 at baseline to 63.9 ± 22.4 at 6 months and 64.9 ± 22 at 12 months (Table 20).

Similarly, the SF-36 scores in the control group also showed a significant improvement at 6 months and 12 months compared to baseline level, indicating an improvement in physical functioning, role limitations to physical health, pain, general health, energy/fatigue, social functioning, role limitations due to emotional problems and emotional well-being. A Welch's t-test was performed to determine if there was a statistically significant difference in SF-36 scores between the two groups. It revealed a statistical difference in pain (BP) score (p-value 0.03) and energy/fatigue (VIT) score (p-value 0.04) between the two groups at 6 months post-surgery. These findings suggest that the significant higher scores at 6 and 12 months may primarily be due to an effective replacement surgery, although further research is needed to confirm the effect of arginine on the SF-36 scores.

Table 20: SF-36 score between two groups in different timelines.

ARGININE (n=12)				
	Baseline	**Pre-op**	**6 months**	**12 months**
SF-36 Physical Functioning: (PF)	27.5 ± 11.8	29.1 ± 12.9	61 ± 19.9	46 ± 27.4
SF-36 Role limitations due to physical health: (RP)	22.9 ± 39.1	20.4 ± 30.4	65 ± 41.5	37.5 ± 37.3
SF-36 Pain: (BP)	39.0 ± 26.4	35.8 ± 20.9	70.8 ± 19.9	45.5 ± 28.2
SF-36 General Health: (GH)	57.2 ± 26.2	61.0 ± 22.4	63.9 ± 22.4	64.9 ± 22.0
SF-36 Energy/fatigue: (VIT)	40.1 ± 27.9	50.9 ± 26.6	47.5 ± 10.7	52.0 ± 27.4
SF-36 Social Functioning: (SF)	66.6 ± 32.1	71.6 ± 29.3	80.0 ± 30.2	73.7 ± 24.6
SF-36 Role limitations due to emotional problems: (RE)	75.0 ± 45.2	57.6 ± 47.3	79.9 ± 29.1	69.9 ± 38.8
SF-36 Emotional well-being: (MH)	76.3 ± 18.9	72.1 ± 24.6	82.0 ± 19.3	78.4 ± 20.7
CONTROL (n=24)				
	Baseline	**Pre-op**	**6 months**	**12 months**
SF-36 Physical Functioning: (PF)	21.25 ± 17.8	21.8 ± 17.3	53.5 ± 22.9	54.0 ± 26.6
SF-36 Role limitations due to physical health: (RP)	16.6 ± 34.3	20.4 ± 29.1	62.5 ± 29.4	37.5 ± 34.9
SF-36 Pain: (BP)	33.6 ± 15.6	28.2 ± 11.1	59.4 ± 29.4	51.0 ± 23.4
SF-36 General Health: (GH)	57.6 ± 20.6	68.4 ± 17.9	67.1 ± 19.4	69.5 ± 29.6
SF-36 Energy/fatigue: (VIT)	39.6 ± 29.1	46.1 ± 23.3	56.5 ± 21.4	55.0 ± 23.7
SF-36 Social Functioning: (SF)	58.3 ± 26.7	52.2 ± 24.9	85.6 ± 19.6	76.2 ± 23.2
SF-36 Role limitations due to emotional problems: (RE)	68.1 ± 44.4	71.2 ± 37.2	84.9 ± 30.2	83.3 ± 33.3

SF-36 Emotional well-being: (MH)	75.3 ± 15.6	75.4 ± 16.7	79.6 ± 17.2	81.0 ± 18.0

The results from Table 20 are illustrated in a box plot in Figure 11-18.

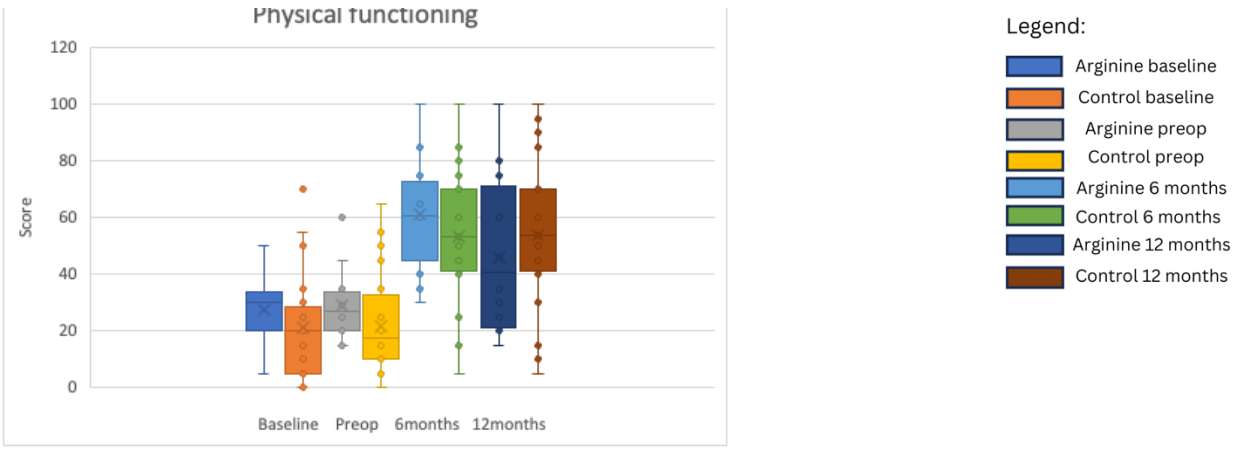


Figure 11: SF-36 score for Physical functioning (PF)

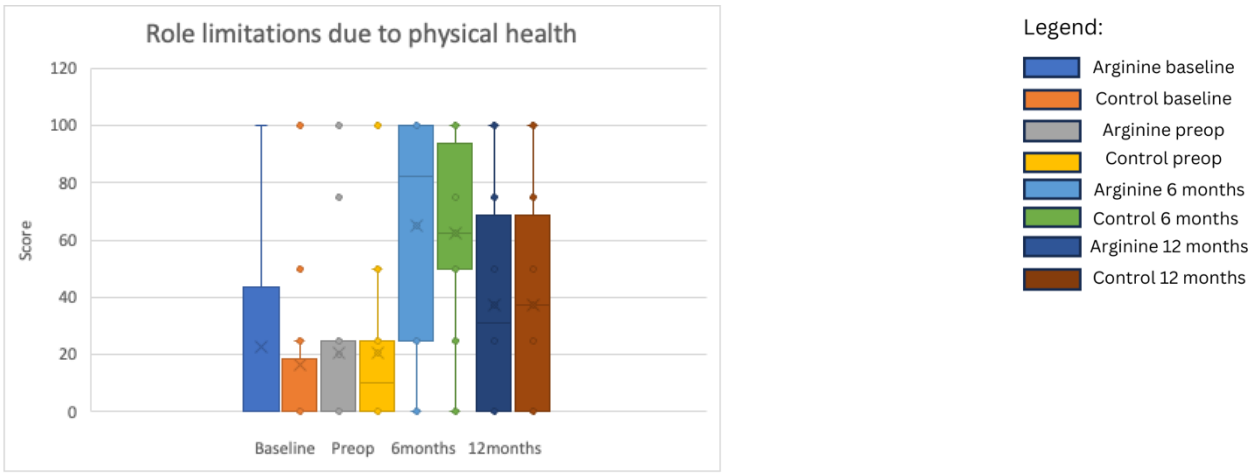


Figure 12: SF-36 score for Role limitations due to physical health (RP)

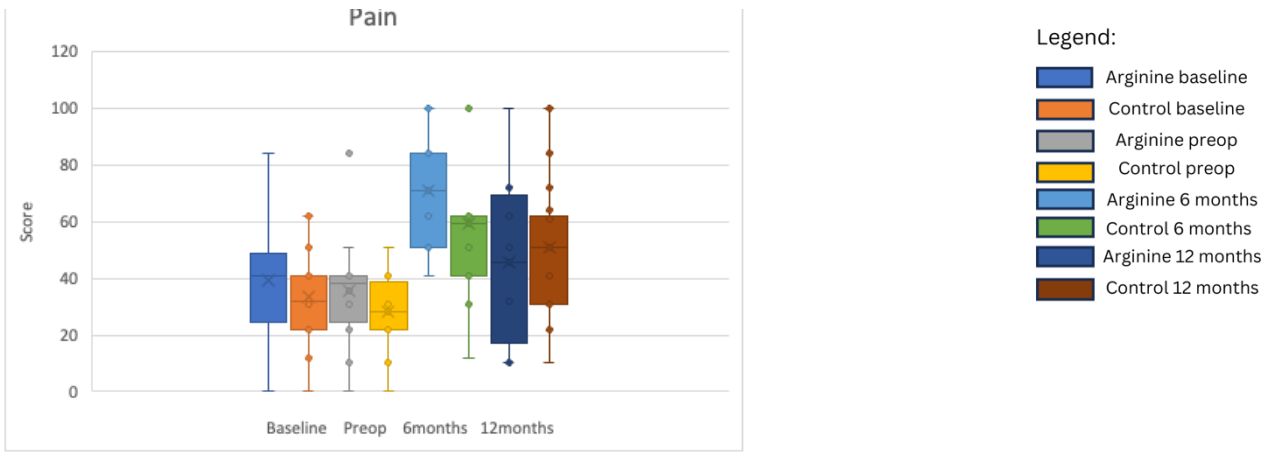


Figure 13: SF-36 score for Pain (BP)

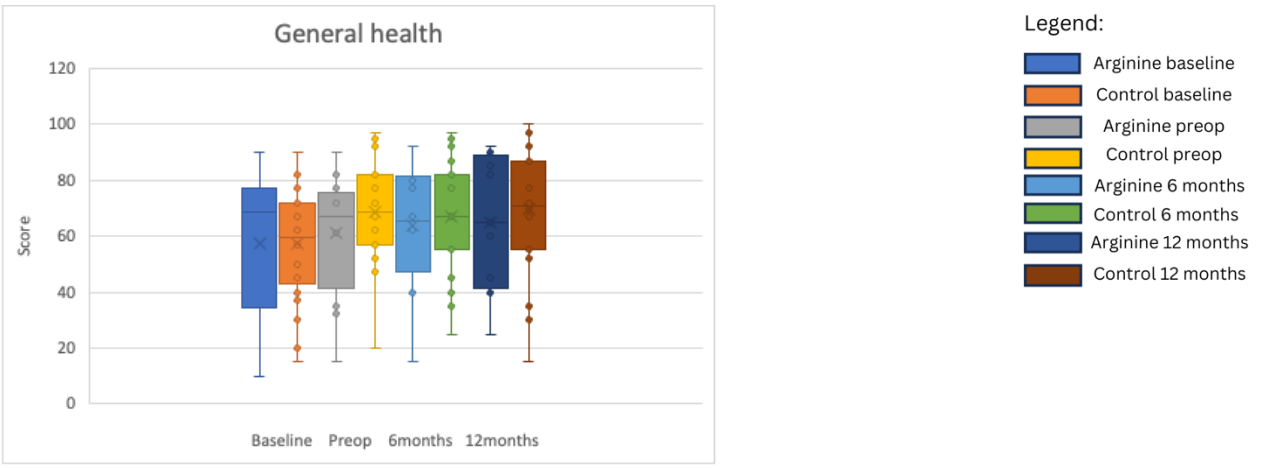


Figure 14: SF-36 score for General health (GH)

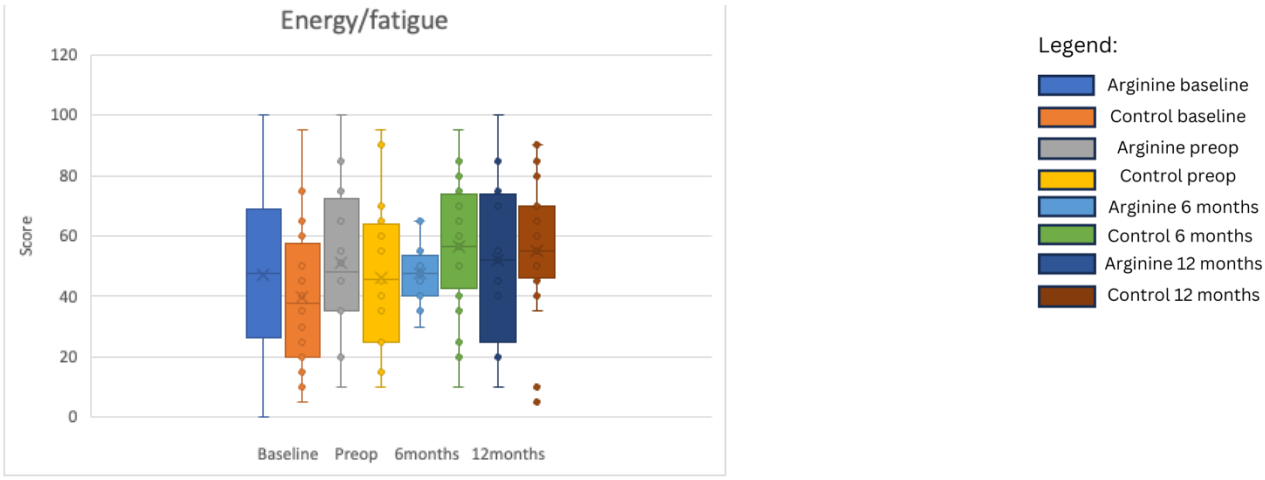


Figure 15: SF-36 score for Energy/Fatigue (VIT)

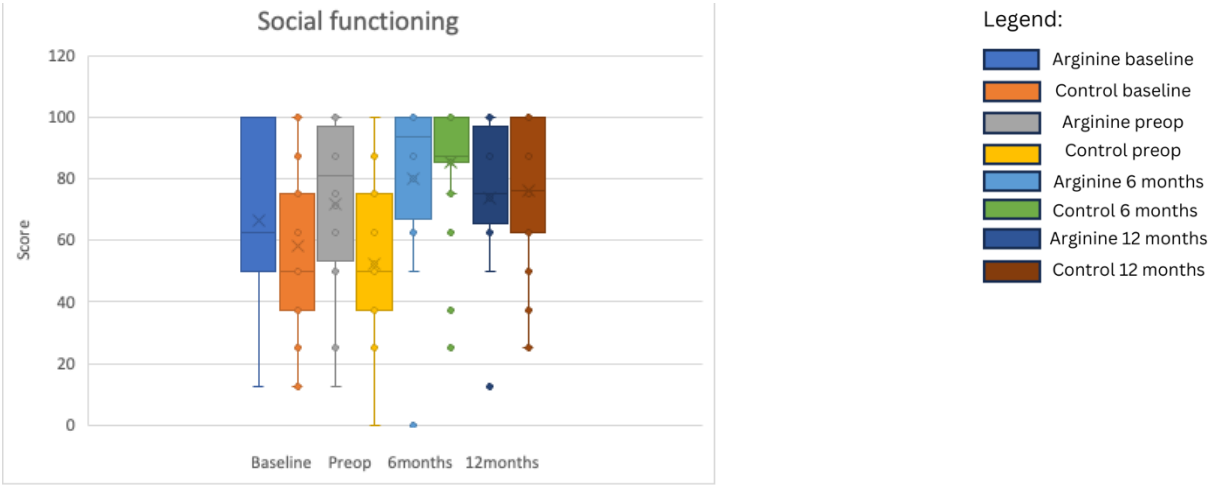


Figure 16: SF-36 score for Social functioning (SF)

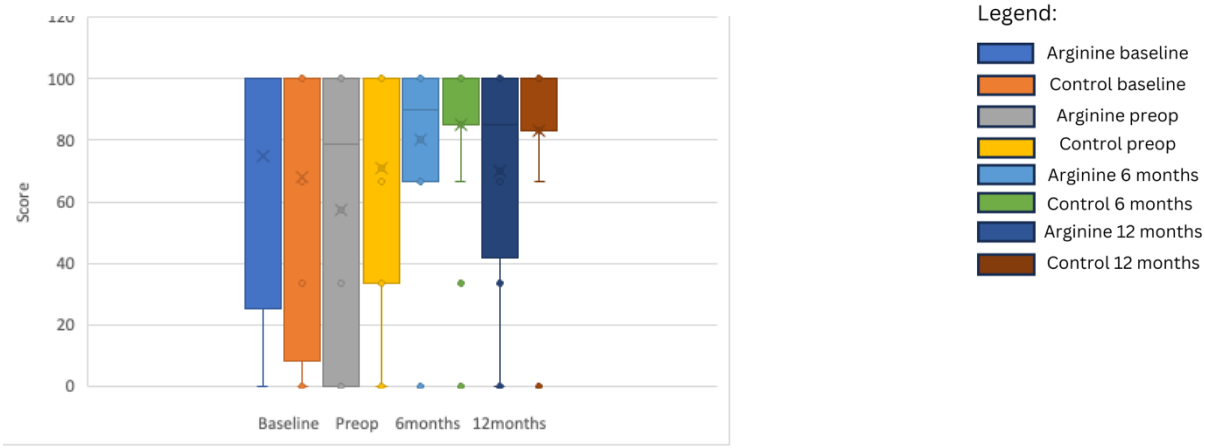


Figure 17: SF-36 score for Role limitations due to emotional problems (RE)

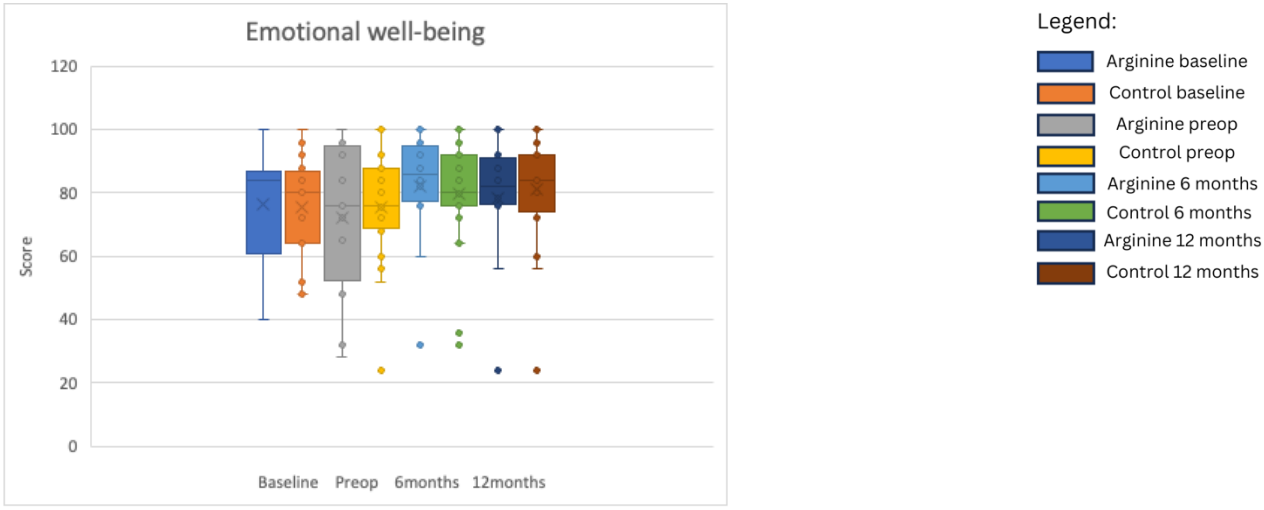


Figure 18: SF-36 score for Emotional well-being (MH)

The SF-36 scores from the eight different variables can be translated to two summary scores: physical component score (PCS) and mental component scores (MCS). The PCS takes into account four variables; they are Physical functioning (PF), Role limitation owing to physical health problems (RP), Bodily pain (BP), General health perception (GH). The MCS takes into account the remaining four variables; they are Vitality (VIT), Role limitation owing to emotional problems (SF), Social functioning (RE) and Mental health (MH). The summary scores are calculated in three different ways in literature; Orthogonal, Oblique and RAND-36. The findings are listed in Table 21.

Table 21: Orthogonal, Oblique and Rand-36 PCS and MCS in both groups.

ARGININE (n=12)				
	Baseline	**Pre-op**	**6 months**	**12 months**
Orthogonal PCS	29. 2 ± 7.7	30.9 ± 7.8	40.5 ± 11.7	34.6 ± 8.9
Oblique PCS	34.7 ± 9.5	35.1 ± 8.4	43.4 ± 8.5	41.3 ± 10.3
RAND-36 PCS	32.8 ± 8.8	32.9 ± 8.1	45.4 ± 9.7	39.2 ± 10.6
Orthogonal MCS	54.8 ± 14.3	52.2 ± 15.3	52.6 ± 12.4	54.1 ± 12.7
Oblique MCS	46.2 ± 11.9	45.1 ± 12.1	49.0 ± 8.2	49.2 ± 11.5
RAND-36 MCS	46.1 ± 13.6	45.4 ± 13.9	50.8 ± 10.4	48.0 ± 12.8
CONTROL (n=24)				
	Baseline	**Pre-op**	**6 months**	**12 months**
Orthogonal PCS	27.2 ± 7.0	28.1 ± 7.5	40.7 ± 9.7	37.4 ± 11.1
Oblique PCS	32.2 ± 7.7	33.2 ± 6.6	44.8 ± 8.2	41.5 ± 9.9
RAND-36 PCS	30.9 ± 7.3	32.2 ± 6.4	43.3 ± 8.7	40.4 ± 10.3
Orthogonal MCS	52.8 ± 10.4	53.2 ± 9.8	55.5 ± 10.5	55.5 ± 10.3
Oblique MCS	43.9 ± 8.6	45.1 ± 7.9	50.8 ± 8.5	49.9 ± 9.3
RAND-36 MCS	43.2 ± 9.8	43.4 ± 9.1	51.7 ± 9.7	50.1 ± 10.6

Based on the data in the Table 21, the baseline HRQOL scores between the arginine supplementation and the control group were similar for the Oblique and Rand-36 methods, but different for the Orthogonal method. For the arginine supplementation group, the baseline Oblique PCS is at 34.7 ± 9.5 which is similar to RAND-36 PCS at 32.8 ± 8.8. The Orthogonal PCS is different with a score of 29.2 ± 7.7 at baseline. The trend is similar for MCS for the arginine supplementation group with the baseline Oblique MCS is at 46.2 ± 11.9 which is similar to RAND-36 PCS at 46.1 ± 13.6. The

Orthogonal MCS is different with a score of 54.8 ± 14.3 at baseline. Similar trend is also observed in the control group for both PCS and MCS. However, after total knee replacement surgery, both groups showed improvements on all outcome measures at the 6-month and 12-month time points, compared to their respective baseline score.

For the arginine group, the PCS score increased 38.7%, 25.1% and 38.4%, for Orthogonal, Oblique and Rand-36 score, respectively from baseline to 6 months. At 12 months the Orthogonal score had increased 18.5%, Oblique score 19% and the Rand-36 score 19.5%. This trend was also found in the control group, the PCS score increased 83.3%, 39.1% and 40.1% for Orthogonal, Oblique and Rand-36 score, respectively from baseline to 6 months. At 12 months the Orthogonal score had increased 68.5%, Oblique score 28.9% and the Rand-36 score 30.7%. The MCS scores in both the groups between Orthogonal, Oblique and Rand-36 scores varied in the different time points.

5.4 Further analysis

5.4.1 Mean time interval for arginine supplementation intake

The mean time interval for arginine supplementation intake was calculated for the individuals who had their arginine concentrations in blood measured. Due to varied scheduling of total knee replacement surgeries for each participant, the duration of arginine supplementation varied between individuals, as reflected in Table 22. The mean time interval for arginine supplementation intake was 179 ± 150 days. CT008 had surgery in both of his right and left knee. Date from time of arginine supplementation intake to his first surgery (RK) was taken into account as good RNA was extracted from RK tibial sample. However, the tibial sample obtained from the left knee surgery did not yield good

RNA, and thus the date of arginine supplementation intake was not considered for this surgery.

Table 22: Study participants with the number of days they took the supplements for.

Arginine supplementation (n=9)	
Study ID	Days they took supplements for (days)
CT002(RK)	111
CT002(LK)	375
CT005	160
CT008	31
CT009	478
CT010	188
CT025	122
CT026	48
CT034	102

5.4.2 Correlation between arginine supplementation days and the change of arginine concentration between before and after supplementation

The correlation between arginine supplementation days and the change of arginine concentration between before and after supplementation was found using spearman correlation test. Table 23 presents data on changes in arginine concentration levels, expressed as a percentage change. Baseline arginine concentration levels were compared to preoperative levels to determine the percentage change. The time interval between baseline and preoperative measurement is also provided.

Table 23: Baseline and Preop arginine concentration levels with percentage change and arginine supplementation days for participants.

StudyID	Baseline (µMol)	Preop (µMol)	% Change	Time interval (Days)
CT002(RK)	64.9	72.5	11.7	111
CT002(LK)	64.9	97.2	49.8	375
CT005	82.3	91.8	11.5	160
CT008	65.1	110	69	31
CT009	57.5	63.3	10.1	478
CT010	67.5	85.6	26.8	188
CT025	36	45.8	27.2	122
CT026	67.9	69.2	1.9	33
CT034	95.8	99.4	3.8	102

For study participant CT002, arginine concentration levels in the right knee (RK) increased by 11.7% from a baseline level of 64.9 to a preoperative level of 72.5, with a time interval of 111 days. However, arginine concentration levels in the left knee (LK) increased by 49.8% from a baseline level of 64.9 to a preoperative level of 97.2, with a longer time interval of 375 days.

Study participant CT005 showed an 11.5% increase in arginine concentration levels, from a baseline of 82.3 to a preoperative level of 91.8, over a time interval of 160 days. CT008 showed the greatest increase in arginine concentration levels, with a 69% increase from a baseline level of 65.1 to a preoperative level of 110, over a shorter time interval of 31 days.

Arginine concentration levels in study participant CT009 increased by 10.1% from a baseline level of 57.5 to a preoperative level of 63.3, over a long-time interval of 478 days. Similarly, in CT010, arginine concentration levels increased by 26.8% from a baseline level of 67.5 to a preoperative level of 85.6, over a time interval of 188 days. In study participant CT025, arginine concentration levels increased by 27.2% from a baseline level of 36 to a preoperative level of 45.8, over a time interval of 122 days. CT026 showed a minimal increase of 1.9% in arginine concentration levels, from a baseline level of 67.9 to a preoperative level of 69.2, over a shorter time interval of 33 days. Finally, in CT034, arginine concentration levels increased by 3.8% from a baseline level of 95.8 to a preoperative level of 99.4, over a time interval of 102 days. Spearman correlation test was performed as the data was not normally distributed. The correlation coefficient was found 0.067 (p-value 0.88) indicating that there is no linear relationship between the two variables, and any observed relationship is likely due to random chance.

5.4.3 Expected VS tablets taken

The 9 individuals who took arginine supplementation were instructed to take 3 tablets per day, however the number varied between every individual from 2-4 tablets per day.

Table 24: Expected VS tablets taken

Study ID (n=9)	Days taken for	Tablets taken	Expected (3 tabs each day)	Average tabs taken each day
CT002(RK)	**111**	414	333	**4**
CT002(LK)	**375**	1189	1125	**3**
CT005	**160**	468	480	**3**
CT008	**31**	112	93	**4**

CT009	**478**	1056	1434	**2**
CT010	**188**	331	564	**2**
CT025	**122**	265	366	**2**
CT026	**48**	149	144	**3**
CT034	**102**	314	306	**3**

Table 24 shows the number of days the individuals took arginine supplementation, the number of tablets taken, the expected number of tablets taken based on a regimen of 3 tablets per day, and the average number of tablets taken each day for the nine individuals in the study. For CT002(RK), he took 414 arginine supplementation tablets for 111 days, which was more than the expected 333 tablets based on 3 tablets per day, with an average of 4 tablets taken per day. For CT002(LK), he took 1189 tablets in 375 days which is less than the expected 1125 tablets to be taken, averaging to about 3 tablets taken per day. CT005 took a total of 468 tablets over 160 days, averaging to 3 tablets per day, which was less than the expected 480 tablets. For CT008, the supplementation was taken for 31 days and a total of 112 tablets were taken, which was more than the expected 93 tablets, with an average of 4 tablets taken per day. For CT009, the supplementation was taken for 478 days and a total of 1056 tablets were taken, which is less than the expected 1434 tablets, with an average of 2 tablets taken per day. CT010 took supplementation for 188 days and a total of 331 tablets, which was less than the expected 564 tablets, with an average of 2 tablets taken per day. CT025 took 265 arginine supplementation tablets in 122 days, which is less than the expected 366 tablets, with an average of 2 tablets taken per day. For CT026, the supplementation was taken for 48 days and a total of 149 tablets were taken, which was more than the expected of 144 tablets, with an average of 3 tablets

taken per day. Finally, for CT034, the supplementation was taken for 102 days and a total of 314 tablets were taken, which is more than the expected 306 tablets, with an average of 3 tablets taken per day. So, we can see 4 out of the 9 individuals took around 3 tablets per day according to our regime, the rest either took less or more, which might influence the arginine concentrations and gene expressions in those individuals.

Table 25: Participants who took 3 or less and more than 3 tablets and their arginine concentrations and gene expression levels.

Variables		**Took 3 or less tablets a day**							**Took more than 3 tablets a day**	
STUDY ID		CT002(LK)	CT005	CT009	CT010	CT025	CT026	CT034	CT002(RK)	CT008
ARGININE CONCENTRATION (µMol)		49.8	11.5	10.1	26.8	27.2	1.9	3.8	11.7	69
GENE EXPRESSION (RQ value)	*MMP13*	19.42	Very high *MMP13*, Excluded for gene expression analysis	0.83	All unaffected cartilage	All unaffected cartilage	10.57	All unaffected cartilage	0.79	All unaffected cartilage
	CTSB	1.77		0.44			1.92		0.75	
	CTSK	1.89		0.48			1.96		1.44	
	ACAN	0.58		0.37			1.55		0.5	
	COL2A1	1.16		1.16			0.6		0.63	

The 9 individuals who had their arginine concentrations measured were divided into two groups. One taking 3 or less tablets a day and another who took more than 3 tablets a day. Not all individuals who had their arginine concentrations measured had gene expression data. From the group which took 3 or less tablets a day, CT005 gave very high *MMP13* values and were later excluded for analysis as an outlier. From the same group, CT010, CT025 and CT034 all had their cartilage samples extracted from unaffected site; thus, these samples had no RNA extracted from them and no gene expression data. CT008 was

from the group who took more than 3 tablets per day and did not have gene expression data as he had his cartilage sample extracted from unaffected site of the knee, thus his collected samples had no RNA extracted from them (Table 25). On average the RQ value for the individuals who took 3 tablets or less (CT002(LK), CT009, CT026) was 10.3 for *MMP13*, 1.4 for *CTSK*, 1.4 for *CTSB*,0.8 for *ACAN* and 1 for *COL2A1*. As per Table 25, in comparison to the individual CT002(RK) who took more than 3 tablets a day, the *MMP13, CTSB, ACAN and COL2A1* RQ value was lower to those who took 3 tablets or less per day. This could be explained due to the duration of supplementation intake in Table 26.

Table 26: Arginine concentration and gene expression levels compared between CT002 in both RK and LK.

Study ID	**Days**	# Tabs taken	ARGININE CONCENTRATION	GENE EXPRESSION				
				MMP13	*CTSB*	*CTSK*	*ACAN*	*COL2A1*
CT002(RK)	**111**	414	11.7	0.79	0.75	1.44	0.5	0.63
CT002(LK)	**375**	1189	49.8	19.42	1.77	1.89	0.58	1.16

Table 26 shows the difference in arginine concentration and gene expression levels in the same individual CT002 in both his right and left knee. The arginine concentration is almost four times higher at 49.8 µMol for CT0002(LK) than 11.7 µMol for CT002(RK). The gene expression levels for genes *MMP13, CTSK, CTSB, ACAN and COL2A1* are also higher for CT002(LK) with RQ values at 19.42, 1.77, 1.89, 0.58 and 1.16 respectively. This trend could be explained due to the longer duration of arginine supplementation intake and number of tablets taken.

5.4.4 Correlation between Arg concentration and RNA seq data

In the study conducted, three genes were investigated for their correlation between arginine concentration and the RNA seq data that was found using spearman correlation test. These genes are *CTSK*, *CTSB*, and *ACAN*. For most individuals, the genes *MMP13* and *COL2A1* were unexpressed in blood and gave unidentifiable levels of transcripts which is why they were not analysed to check the correlation. The Spearman correlation coefficient was calculated to measure the strength and direction of the relationships, while the p-value was used to determine the statistical significance of the correlations.

Firstly, the gene *CTSK* showed a positive Spearman correlation coefficient of 0.31, indicating a weak positive, however, the corresponding p-value of 0.11 suggests that this correlation is not statistically significant.

Secondly, the gene *CTSB* exhibited a weak negative correlation with the trait, as indicated by a Spearman correlation coefficient of -0.06. The associated p-value of 0.41 further supports the notion that this correlation is not statistically significant.

Lastly, the gene *ACAN* also demonstrated a weak negative Spearman correlation coefficient of -0.08. Similar to the previous genes, the corresponding p-value of 0.38 suggests that this correlation is not statistically significant as per Table 27.

Table 27: Correlation between Arginine concentration and RNA seq data.

Gene	**Spearman correlation coefficient**	**P-value**
CTSK	0.31	0.11
CTSB	-0.06	0.41
ACAN	-0.08	0.38

5.5 Adverse effects

According to the most recent research studies, 1.5 g of L-arginine daily has no adverse effect. Taking more than 1.5g of arginine daily might cause nausea, diarrhea, abdominal pain, bloating, gout, allergic response, airway inflammation, or worsening of asthma symptoms. L-arginine supplementation is not recommended for individuals who have recently experienced a heart attack, as there are concerns that it may increase the risk of death. Caution should be exercised when using L-arginine if an individual has allergies or asthma, as it can potentially exacerbate these conditions. Additionally, individuals with a history of cold sores or genital herpes should be cautious, as excessive amounts of L-arginine in the system may potentially trigger the virus responsible for these conditions [139]. All the side effects are relatively low. While there is no published data on how many patients would experience these side effects, for our study, some patients experienced side effects like nausea, constipation, congestion, cold-like symptoms, and stress. These side effects may not be due to the supplementation but due to other causes like COVID-19, as the dosages used in this clinical trial are way below the tolerable upper intake levels approved by Health Canada. The adverse effects were noted by the research assistant and not addressed as urgent medical concerns.

6 Discussion and Limitation

We conducted a randomized clinical trial aimed at evaluating the effect of oral L-arginine supplementation on knee OA in patients scheduled for total knee replacement surgery due to primary knee OA.

An ideal approach for clinical trials is a randomized double-blind placebo-controlled study, but in this trial, the study design is randomized and controlled, with an open-label 2-arm format. For this study, it is more practical to not use a placebo as it would be difficult to produce placebos. Most of the clinical trials conducted around the world that have placebos are conducted by pharmaceutical companies. Also, because our primary outcome is objective; we are looking into the molecular level by measuring gene expression level. The primary outcome is not subjective as it would have had significant placebo effect. The open-label format of this study promotes transparency and credibility as both the investigator and participants are aware of the treatment being utilized, minimizing bias, but not eliminating it.

Considering this is a pilot clinical trial, we intended to enroll a relatively small sample size of about 100 individuals, with 50 assigned to each group. Limited funding was also a factor in determining thee sample size during study design.

Finally, a sample of 49 participants aged between 40- 80 years old, with an approximately equal gender distribution, was included in the trial from a single center from a population

of St. John's, Newfoundland. Relying solely on recruitment from a single center has presented several complications, such as limited sample size, insufficient diversity amongst participants, and difficulties in meeting recruitment goals. To address such obstacles, investigators need to modify their recruitment strategies, collaborate with other centers, or broaden the inclusion criteria for the study. Multicenter recruitment will further enhance generalizability. To achieve successful recruitment in clinical trials, recruiting from multiple centers and implementing a well-designed strategy to identify and attract potential participants is often necessary. In future studies it could be taken into consideration to include not only Caucasians as we did in this pilot clinical trial, but other populations from around the world.

Out of the 380 individuals approached, only 49 participants agreed to participate, resulting in a low response rate of 13%. This could be because of the concern of the participants regarding the safety of the supplements and their concerns over its efficacy. Patients may have individual concerns about various adverse effects that may not align with the concerns of the physicians. However, the trial still managed to collect valuable data from the willing participants.

After randomization, 48 individuals were included in the trial, with 24 assigned to receive arginine supplementation and 24 as control: receiving nothing. However, one participant from the supplementation group was later excluded due to rheumatoid arthritis, resulting in 23 individuals receiving supplementation. One individual from the control group was excluded as no affected tibial sample was collected. Eleven individuals withdrew their

consent, all of whom were from the supplementation group. Their reason for withdrawal varied from experiencing symptoms like constipation and cold-like symptoms to finding it stressful to take the tablets. This impacted the results as it reduced the sample size further to a smaller number to show significant effects.

For descriptive analysis, 36 individuals were included, 12 receiving arginine supplementation and 24 as controls. There was no statistical difference seen in age, sex, and BMI between the two groups.

In this study, metabolomic profiling was performed to determine arginine concentration levels in blood samples collected at baseline and preoperative stages. It was found that there was no significant difference in the percentage change between the two groups ($p=0.76$). Even though there was an increase in arginine concentration from baseline to pre-op, this increase occurred in both the groups which received supplementation and did not receive supplementation. This might conclude that, even with arginine supplementation from an external source, arginine might still be used up or metabolized into other products to maintain a relative stable level in circulation. It can also be due to a possible chance of contamination of arginine from an external source for the control group primarily from diet - fish, red meat, poultry, whole grains, etc.

In previous literature, it was found through a metabolomics approach that arginine deficiency was associated with OA [140]. A novel and promising result were found, which showed plasma concentration of arginine in knee OA patients to be, on average, 69 μM lower than that in non-OA controls. So, we tested the disease-modifying effects of

arginine supplementation on human articular cartilage by regulating cartilage maintenance and repair genes. The genes of interest were on cartilage degradation enzymes - *MMP13* (matrix metallopeptidase 13), *CTSK* (cathepsin K), *CTSB* (Cathepsin B), which we previously found overexpressed in OA-affected cartilage, and cartilage syntheses genes - *ACAN* (aggrecan) and *COL2A1* (type II collagen), which we previously found down expressed in OA-affected cartilage in previous literature [135].

We did not find any statistical differences in the gene expression of these five genes in cartilage between the arginine supplantation group and the controls. However, *MMP13* expression was higher in the arginine group (5.21 ± 7.18) compared to the control group (2.57 ± 4.23). These findings suggest that arginine supplementation may have a moderate effect on increasing *MMP13* expression in OA-affected cartilage. The effect is opposite to what we expected, suggesting that arginine supplementation might have a detrimental effect on cartilage. A previous study of mouse OA model documented an increased expression of *MMP13* in cartilage by an increase arginase-II which is one of the key enzymes to metabolize arginine in tissues[141]. Thus, we could postulate that arginine supplementation might induce a higher expression of arginase in cartilage which in turn leads to a higher expression of *MMP13* and play a role in OA pathogenesis. Further studies are needed to test this hypothesis.

In this study, a self-administered questionnaire (WOMAC and SF-36) was employed as means to assess the health outcomes of the participants. The self-administered approach allowed for the collection of detailed and subjective information directly from the participants, enabling a comprehensive evaluation of their health status. This method

proved to be a convenient and efficient way to gather data on health outcomes, as it minimized potential biases that could arise from direct observation or interviewer influence.

For WOMAC scores, at baseline, there were no significant differences in WOMAC pain, stiffness, function, and total scores between the arginine supplementation group and the control group. At 6 months and 12 months, both groups showed significant improvements in all WOMAC measures compared to baseline, indicating an improvement in knee pain, stiffness, and function. This could be explained primarily due to the surgical treatment itself, which might have had an impact on the health status of the participants. The arginine supplementation group had significantly lower WOMAC scores at 6 months and 12 months compared to baseline, indicating a greater improvement in knee symptoms compared to the control group. Some patients may experience substantial improvement within the first 6 months after surgery, others may continue to experience improvement beyond this timeframe. Ultimately, the reasons why patients tend to do better at 6 months, rather than 12 months post-surgery, may vary depending on a variety of factors, the individuals' overall health and well-being, the quality of aftercare, age, physical activity, underlying medical conditions, and adherence to post-operative instructions. Additionally, recovery from surgery is a complex process that often involves multiple stages, and patient outcomes may vary depending on the stage of recovery being considered. However, further research is needed to confirm the specific effect of arginine on WOMAC scores.

For SF-36 scores, there were no significant differences in most SF-36 dimensions between the arginine supplementation group and the control group. The only significant difference was found in the Physical Functioning (PF) dimension, where the arginine group had a slightly higher mean score. At 6 months and 12 months, both groups showed significant improvements in all SF-36 measures compared to baseline, indicating an improvement in various aspects of health-related quality of life. The arginine supplementation group had significantly higher SF-36 scores at 6 months and 12 months compared to baseline, indicating a greater improvement in physical functioning, role limitations, pain, general health, energy/fatigue, social functioning, role limitations due to emotional problems, and emotional well-being compared to the control group. However, further research is needed to confirm the specific effect of arginine on SF-36 scores.

The study further calculated summary scores for the physical component (PCS) and mental component (MCS) using different methods (orthogonal, oblique, RAND-36). The results showed improvements in both PCS and MCS scores for both groups at 6 months and 12 months compared to baseline. The arginine supplementation group generally had higher summary scores, indicating a greater improvement in overall physical and mental health-related quality of life which might be primarily explained due to successful surgery; further studies are needed to find the effect of arginine specifically.

In our study, we had anticipated that each patient would take three tablets daily; however, this varied among individual participants. To determine adherence levels, we indirectly measured adherence by counting the remaining pills in the medication containers that were returned by each participant. By comparing the actual count of remaining tablets with the expected number of tablets taken, we were able to estimate the overall level of

adherence. It is important to note that this method does not definitively confirm that participants in the active group ingested the tablets, as they may have discarded or misplaced them.

To improve adherence in clinical trials, it is crucial to establish open and ongoing communication with participants, provide them with adequate education about the trial, and foster a trusting relationship. These measures can encourage honest reporting and help ensure that participants adhere to the trial requirements as closely as possible.
The participants exhibited a variability in the duration of supplement intake, deviating from the originally intended 6-month supplementation plan. The period of arginine supplementation ranged from 1 month to 16 months, reflecting differences in the timing and scheduling of the patient's total knee replacement surgeries, primarily attributed to the COVID-19 pandemic-related delays and rescheduling.

A Spearman correlation test was performed to investigate the association between the duration of arginine supplementation days and the change in arginine concentration before and after supplementation. The obtained correlation coefficient was 0.067 with a p-value of 0.88, indicating a lack of significant relationship between the duration of arginine supplementation days and the change in arginine concentration. These results were unexpected and may suggest variations in the absorbability of arginine in the bloodstream among individuals or potential conversion of arginine into other metabolites. Further research is necessary to explore these factors in greater depth.

The number of days of supplementations was compared with the arginine concentration and gene expression levels in one individual (CT002) who had surgery on both his knees. The arginine concentration was found to be four times higher after left knee surgery than it was for the right knee. The gene expression levels were also significantly found to be a few times higher for cartilage from the left knee than the right knee. Even though this participant took higher numbers of tablets before his surgery for his right knee than the left knee, this observed trend could be attributed to the longer duration of arginine supplementation intake and the number of tablets consumed.

The correlation between arginine concentration and RNA seq data was examined for three genes, *CTSK*, *CTSB*, and *ACAN*, which had identifiable transcript levels in their blood sample. There were no statistically significant correlations between arginine concentration and the investigated genes. This could again be explained due to the small sample size, a larger sample is needed to find a better correlation.

It is crucial to highlight that these conditions are derived from the specific data and statistical analysis used in this study, and their applicability to other populations or research contexts may vary. Further investigation and replication of these findings are necessary to obtain a more comprehensive understanding of the potential connections between arginine and these genes.

When planning and conducting clinical trials, it is crucial to carefully consider and address patient dropouts to ensure accurate and trustworthy results. To achieve this, a more detailed and comprehensive informed consent process should be implemented to help participants thoroughly comprehend the trial's nature, associated risks and benefits, and the expected time commitment. A thorough informed consent process can help establish realistic expectations for participants and minimize dropouts due to misunderstandings or unforeseen obligations. Furthermore, maintaining regular communication with participants throughout the trial, offering support, responding to queries, and addressing concerns can enhance participant retention and minimize the chance of dropouts.

To accommodate the dropouts in the study, a sensitivity analysis could be performed to compare different outcomes of the trial under varying assumptions regarding the missing data from patients who withdrew their consent. Statistical methods could also be utilized to estimate the missing data through imputation. For future studies, when designing the study, the drop out rate should be considered as a part of the sample size for a sample size calculation.

In the future when a larger study is planned, it is advisable to conduct an interim safety analysis to ensure patient safety and reduce the risk of harm. The interim safety analysis can be conducted when approximately half of the data has been collected, which provides enough information to assess the safety of arginine and make any necessary adjustments to the study protocol, such as modifying the dose or discontinuing the study altogether.

This will serve as a valuable tool to monitor the trials safety profile and identify any potential safety concerns early on. It can also act as a guide to optimize the sample size of the larger trial, ultimately enhancing the overall feasibility of the study and minimizing safety issues.

It is important to note that the study had a relatively small sample size due to insufficient enrollment and patient dropouts and is an underpowered clinical trial. Studies with a larger sample size may be necessary to validate these patterns. Furthermore, additional factors such as dosage, the duration of supplementation, as well as the baseline health status of the participants could also impact the results.

Assessing the clinical benefit of a drug in the context of a surgical intervention can be quite challenging. The surgical procedure can itself have an extensive impact on the patient's health outcomes, making it difficult to separate the effects of the drug from those of the surgery. Furthermore, surgical outcomes are often measured in terms post-surgical health outcome, which may be influenced by a variety of factors beyond the drug intervention. Self-reported health outcome would primarily be reported due to the effect of a successful surgical intervention like TKR; therefore, it can be difficult to isolate the effects of a drug. Evaluating the clinical benefit of a drug in the context of a surgical intervention requires careful consideration of these and other challenges and may require innovative trial designs or statistical methods to address them effectively.

Also, most disease modifying agents are used early in OA to slow down the progression. At end stage OA, there is significant structural damage, and DMOADs are not that

helpful as the pain is mostly mechanical in origin. Focus given to this disease at an early stage might be more useful as it could help with the structural progression of the disease.

Another limitation of the study was the COVID-19 outbreak. The COVID-19 outbreak was first reported in the Chinese city of Wuhan in December 2019. The COVID-19 outbreak has had a severe impact on the field of research. A lot of research was suspended and focused on research on COVID-19 and other essential research. On January 30, 2020, the World Health Organization (WHO) declared the outbreak as a public health emergency of international concern (PHEIC), and on March 11, 2020, it was classified as a pandemic. Not just by taking lives, this novel virus has significantly taken a toll on the economic status of the whole world, employment, and, more importantly, the physical and mental health of the world population. During the pandemic, respondents reported fewer problems in the pain/discomfort dimension (64.0% vs. 51.6%) and more problems in the usual activities (26.0% vs. 40.5%) and anxiety/depression dimensions (37.2% vs. 69.9%) [143].

The COVID-19 pandemic has had a significant impact on clinical research worldwide. Various aspects of clinical trials and research studies have been affected, resulting in delays, modifications, and challenges in conducting research. This clinical trial was also interrupted due to the lockdown, and most of the scheduled knee replacement surgeries of the patients got delayed further due to overwhelmed healthcare systems. This disrupted the original plan of the study planning to see the effects of arginine supplementation for 6 months. To adapt to social distancing measures and to reduce exposure risks, data collection and monitoring of the patients were also hampered as the patients were also

hard to reach. However, remote monitoring over telephone interviews helped maintain patient engagement and data collection for the individuals who participated in the study. Blood collection and the date of the patient's knee replacement surgeries were pushed back to a later date when surgeons scheduled such elective surgeries. Some patients are still yet to have their surgeries.

7 Conclusion

7.1 Recent development

Regenerative therapies for osteoarthritis (OA) have garnered significant interest in recent years due to their ability to potentially address more than just temporary symptom relief. These innovative treatments aim to promote cartilage repair and regeneration within the affected joint, going beyond traditional approaches. Currently undergoing clinical trials, these investigational disease-modifying osteoarthritis drugs (DMOADs) target specific mechanisms of the disease with the ultimate goal of preserving or restoring articular cartilage. It is important to note that these drugs and biologics are still in various stages of clinical development and have not yet received approval from the FDA [144]

Novartis, a leading pharmaceutical company, has received Fast Track designation from the US Food and Drug Administration (FDA) for LNA043, a potential treatment for knee osteoarthritis. This designation is granted to advance the development and review of therapies that aim to address unmet medical needs. LNA043 is an investigational, small interfering RNA (siRNA) therapy designed to target and inhibit a specific gene associated

with the progression of osteoarthritis. The Fast Track designation reflects the FDA's recognition of the potential benefits of LNA043 in addressing the challenges faced by patients with osteoarthritis, a degenerative joint disease that affects millions of people worldwide. Novartis will work closely with the FDA to accelerate the clinical development and regulatory review process of LNA043 to bring this innovative therapy to patients in need as quickly as possible [145].

QUC-398 is an investigational drug developed by Novartis for the treatment of osteoarthritis. It is a selective Janus kinase inhibitor that aims to provide relief from pain and inflammation associated with the disease. QUC-398 is administered through the subcutaneous route, allowing for convenient self-administration by patients. Clinical trials have shown promising results, with the drug demonstrating significant improvements in pain and physical function compared to a placebo. Novartis has submitted a New Drug Application (NDA) to the US Food and Drug Administration (FDA) seeking approval for QUC-398. The likelihood of approval for QUC-398 is estimated to be high, with positive clinical trial data and an unmet need for effective osteoarthritis treatments. If approved, QUC-398 has the potential to provide a valuable therapeutic option for patients suffering from osteoarthritis [146].

Several other promising treatment options are being investigated for osteoarthritis. INVOSSA™ is a cell and gene therapy that combines non-transformed chondrocytes with chondrocytes that have been modified to overexpress TGF-1. It has received approval in South Korea and is undergoing phase 3 clinical trials in the US. MIV-711 is a selective

inhibitor of cathepsin K, which plays a role in joint degradation. Clinical trials for MIV-711 are currently in phase 2. SM04690 is an intra-articular knee injection that targets cellular mechanisms involved in cartilage regeneration by inhibiting the Wnt pathway. It is currently being tested in phase 2 clinical trials. Sprifermin, a recombinant human FGF-18 protein, stimulates chondrocyte proliferation and enhances cartilage growth and repair. It is currently in phase 3 studies [147].

7.2 Future directions

In future studies, it would be beneficial to replicate the trial using a larger sample size. This would increase the number of participants and improve the response rate. Additionally, a larger sample size would minimize the impact of participants withdrawing from the supplementation group. Extending the duration of the study would provide a clearer understanding of the long-term effects of arginine on OA.

To gain a better understanding of the effects of arginine supplementation, it would be advantageous to include a more diverse population, encompassing individuals from different countries. This would allow for a more comprehensive exploration of the potential variations and changes within different populations.

As of May 5th, 2023, the World Health Organization (WHO) no longer considers COVID-19 a public health emergency of international concern [141], [148]. With the healthcare sector gradually returning to normalcy, patients whose surgeries were rescheduled can be included in the trial. Conducting the trial, without the pandemic having its effect is favorable as it won't result in any unprecedented delays.

Contrary to our expectations, our study found that arginine supplementation had an opposite effect on *MMP13* expression, suggesting a potential detrimental impact on cartilage. Previous research in a mouse model of OA demonstrated increased *MMP13* expression in cartilage due to elevated levels of arginase-II, a key enzyme involved in arginine metabolism [141]. Based on this, we can hypothesize that arginine supplementation may induce higher arginase expression in cartilage, leading to increased *MMP13* expression and contributing to the pathogenesis of OA. Further studies are necessary to explore this hypothesis and gain a deeper understanding of the pathways involving arginine and its metabolites in relation to cartilage degradation enzymes and cartilage synthesis genes.

7.3 Conclusion

In conclusion, our data did not show significant differences in cartilage synthesis and degradation genes between arginine supplementation and non-supplementation, which might be due to the small sample size. Thus, from the study findings, arginine supplementation has no disease-modifying effect on human articular cartilage by regulating cartilage maintenance and repair genes. Further studies with a larger sample size are required to verify our findings.

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Appendix

9.1 **Appendix A:** Ethics approval

Liu, Ming

From:	Zhai, Guangju
Sent:	16 September, 2022 11:50 AM
To:	Liu, Ming
Subject:	FW: HREB - Approval of Ethics Renewal 585668
Attachments:	Co-Chair Peter Daley 2022.pdf

From: "administrator@hrea.ca" <administrator@hrea.ca>
Date: Friday, September 16, 2022 at 11:49 AM
To: "Zhai, Guangju" <Guangju.Zhai@med.mun.ca>
Cc: Hreaadministrator <administrator@hrea.ca>
Subject: HREB - Approval of Ethics Renewal 585668

Researcher Portal File #: 20190705

Dear Dr. Guangju Zhai:

This e-mail serves as notification that your ethics renewal for study HREB # 2018.194 – Effects of Vitamin D, Anti-oxidants (vitamin C, E, and beta-carotene) and Arginine on Osteoarthritis: A Pilot Clinical Trial – has been approved. Please log in to the Researcher Portal to view the approved event.

Ethics approval for this project has been granted for a period of twelve months effective from October 24, 2022 to October 24, 2023.

Please note, it is the responsibility of the Principal Investigator (PI) to ensure that the Ethics Renewal form is submitted prior to the renewal date each year. Though the Research Ethics Office makes every effort to remind the PI of this responsibility, the PI may not receive a reminder. The Ethics Renewal form can be found on the Researcher Portal as an "Event".

The ethics renewal [was reviewed by the Health Research Ethics Board at their meeting dated September 15, 2022.

Thank you,

Research Ethics Office

(e) info@hrea.ca
(t) 709-777-6974
(f) 709-777-8776
(w) www.hrea.ca
Office Hours: 8:30 a.m. – 4:30 p.m. (NL TIME) Monday-Friday

9.2 **Appendix B**: General Questionnaire

		/			/				

Faculty of Medicine

Discipline of Genetics
Craig L. Dobbin Genetics Research Centre
St. John's, NL Canada A1B 3V6
Tel: 709-864-6668 Fax: 709-864-6531
www.med.mun.ca

Effects of Arginine on Osteoarthritis: A Pilot Clinical Trial

General Questionnaire

Date form completed: (dd/mm/yyyy)

Instruction for completing the questionnaire:

Please answer all questions to the best of your ability (leave blank if unknown).

Please write in block letters using the boxes where provided.

Use a black/blue pen.

Cross out any mistakes & write correct answers just below the relevant boxes.

Indicate your response by filling in the box next to the most appropriate answer or by writing clearly in the boxes or space provided.

Your answers will be completely confidential.

Self administered: ☐

Research assistant administered: ☐

Name and address

Surname

Given name

Title

Maiden Name (if applicable)

Address

Province

Postal code

Date of Birth (dd/mm/yyyy)
/ /

Place of Birth
City/Town

Province/Country

Gender: Male ☐ Female ☐

MCP number:

Section 1: Demographics

1. Ethnic: White ☐ Black ☐ Other ☐, please specify

2. Height: ☐☐☐ cm

3. Weight: ☐☐☐ kg

4a. Smoker: yes (current) ☐ no ☐ ex-smoker ☐

4b. If Yes, how many cigarettes do/did you smoke a day? ☐☐☐

5a. In the past 4 weeks approximately how many units of alcohol did you drink per week? (1 unit = 1 glass of wine/½ pint of beer /1 shot of sprit)? ☐☐☐

5b. Do you think your drinking habits in the last 4 weeks reflect your typical drinking habit?

Yes ☐

no, less than usual ☐

no, more than usual ☐

6a. How heavy were you when you were born?

☐☐☐ grams or ☐☐ lbs ☐☐ ozs

6b. If weight unknown, were you Light ☐ Average ☐ Heavy ☐

6c. Were you born prematurely (more than 1 week early) Yes ☐ No ☐

7a. How heavy were you at age 20 yrs? ☐☐☐ kg

7b. How heavy were you at age 50 yrs? ☐☐☐ kg

For women only:

8. At what age did your period start? ☐☐

9. At what age did your period stop? ☐☐

Section 1: Demographics (continued)

10a. Have you had a hysterectomy (removal of the womb)? Yes ☐ No ☐

10b. If Yes, how old were you? ☐☐

10c. Did the hysterectomy include removal of the ovaries?

Yes ☐ No ☐ or Unknown ☐

11. Have you ever taken an oral contraceptive pill? Yes ☐ No ☐

12a. Have you ever taken hormone replacement therapy? Yes ☐ No ☐

12b. If Yes, how long in total did you take it for?

Less than 3 months ☐

3 to 12 months ☐

1 to 5 years ☐

Longer than 5 years ☐

13. How many live births have you had? ☐☐

Section 2 - Occupation

14a. What was your current/last occupation (job title)?

14b. In what industry did you carry out this occupation (eg farming, shipyard, car factory, shoe shop, hospital, insurance office)?

14c. Number of years in job: ☐☐

15a. What was the main occupation that you held for the longest period of time (job title)?

15b. In what industry did you carry out this occupation (eg farming, shipyard, car factory, shoe shop, hospital, insurance office)?

15c. Number of years in job: ☐☐

For your main occupation in an average working day, did you:

16. Sit for more than two hours in total? Yes ☐ No ☐ Don't know ☐

17. Stand or walk for more than two hours in total? Yes ☐ No ☐ Don't know ☐

18. Kneel for more than one hour in total? Yes ☐ No ☐ Don't know ☐

19. Squat for more than one hour in total? Yes ☐ No ☐ Don't know ☐

20. Drive for more than 4 hours in total? Yes ☐ No ☐ Don't know ☐

21. Walk more than 2 miles in total? Yes ☐ No ☐ Don't know ☐

Section 2 – Occupation (continued)

22. In the course of your work how often on average did you lift or carry weights of 10 kg or more?

Never ☐

Less than once per week ☐

1 to 10 times per week ☐

More than 10 times per week ☐

23. In the course of your work how often on average did you lift or carry weights of 25kg or more

(Equivalent to half a bag of cement)

Never ☐

Less than once per week ☐

1 to 10 times per week ☐

More than 10 times per week ☐

Section 3 – Medical history (1)

Please list in the box below all medication that the patient is currently taking:

Have you **EVER** been told by a Doctor or other health professional that you have **ANY** of the following conditions (please tick all that apply to you):

Cardiology

24. Congenital Heart Disease ☐
25. Coronary Heart Disease ☐
26. Heart Attack ☐
27. Hypertension (high blood pressure) ☐
28. High Blood Pressure in Pregnancy ☐
29. Angina ☐
30. High Cholesterol ☐
31. Deep Vein Thrombosis ☐
32. Varicose Veins ☐
33. Pulmonary Embolism ☐

Immunology/Chest Medicine

34. Asthma ☐
35. Hayfever ☐
36. Eczema ☐
37. Sinusitis ☐

Gastroenterology/Endocronology

38. Heartburn ☐
39. Irritable Bowel Syndrome ☐
40. Crohn's ☐
41. Diabetes ☐

Neurology/Psychiatry

42. Dyslexia ☐
43. Clinical Depression ☐
44. Anxiety/Stress Disorder ☐
45. Epilepsy ☐
46. Stroke ☐
47. Motion Sickness ☐
48. Migraine ☐

Section 3 – Medical history (2)

Have you **EVER** been told by a Doctor or other health professional that you have **ANY** of the following conditions (please tick all that apply to you):

Oncology/Cancers

49. Breast Cancer ☐
50. Colon Cancer ☐
51a. Skin Cancer ☐ if yes, was it:
51b. Melanoma ☐
51c. Basal Cell Carcinoma ☐
51d. Squamous Cell Carcinoma ☐

Rheumatology

52. Gout ☐
53. Paget's Disease ☐
54. Bunions ☐
55. Frozen Shoulder ☐
56. Osteoporosis ☐
57. Carpal Tunnel ☐
58. Tennis Elbow ☐
59. Golfer's Elbow ☐

Dermatology/Skin

60. Acne (that caused scarring) ☐
61. Viral Warts ☐
62. Cold Sores ☐

Hearing

63. Hearing Loss ☐
64. Tinnitus (ringing in ears) ☐

Opthalmology/Eyes

65. Glaucoma ☐
66. Cataract ☐
67. Myopia (short sightedness) ☐
68. Age-related Macular Degeneration (AMD) ☐

Urology

69. Incontinence (leak urine) ☐
70. Polycystic ovary syndrome ☐

Section 3 – Medical history (3)

Please answer the following questions by ticking the appropriate box:

71a. Have you ever lost the use of an arm, leg, vision, or ability to speak?

Yes ☐ No ☐

71b. If Yes, how long for : less than 24 hours ☐ or more than 24 hours ☐

72a. Do you usually bring up phlegm from your chest in winter? Yes ☐ No ☐

72b. Do you usually bring up phlegm on most days for at least 3 months a year?

Yes ☐ No ☐

73a. Have you had heartburn or acid regurgitation in the last year? Yes ☐ No ☐

73b. If Yes, how many times have you had heartburn/acid regurgitation in the last year?

Less than once a month ☐

About once a month ☐

Once a week or more ☐

74a. Have you been bothered by recurrent headaches? Yes ☐ No ☐

74b. If Yes, do you still have recurrent headaches? Yes ☐ No ☐

74c. If Yes, are your most troubling headaches

One sided ☐

Accompanied by sensitivity to light/noise ☐

4 to 72 hours in duration if untreated ☐

Section 3 – Medical history (4)

Please answer the following questions by ticking the appropriate box:

75. Since turning 16 have you ever fractured or broken a bone? Yes ☐ No ☐

If Yes, please tick which of the following bones you have fractured or broken

Wrist ☐ Arm ☐ Ribs ☐ Hip ☐ Ankle ☐ Vertebra ☐ Other ☐

76. In the past 3 months have you had pain in your back on most days?

Yes ☐ No ☐

If Yes, does this pain typically radiate to either leg? Yes ☐ No ☐

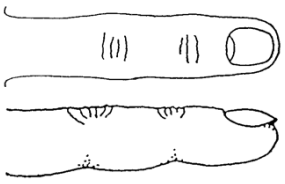
77. In the past 3 months have you had any pain in any part of your body lasting at least 24 hours?

Yes ☐ No ☐

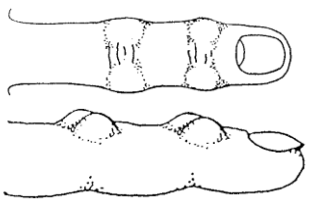
Section 4 – Nodal status

We are interested in knowing whether you have any finger nodes. These sometimes relate to arthritis at the hand and other joints. A finger node is a firm, bobbly swelling on the back of the finger joint.
For example:

A finger **without** nodes:



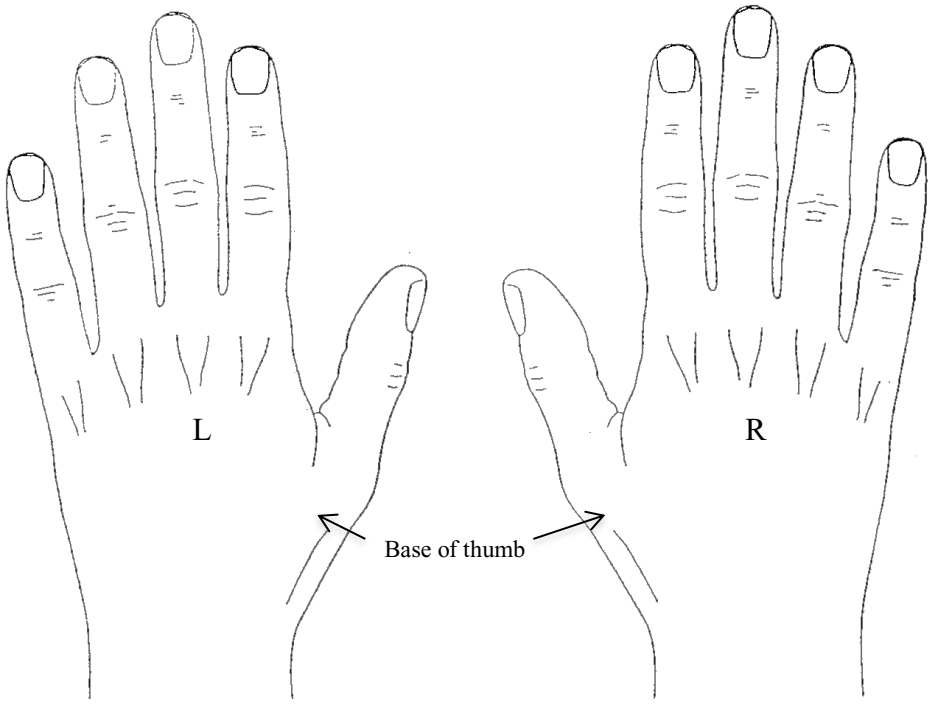
A finger **with** nodes:



When you meet with the research assistant, please look at your hands and then answer the following questions:

78a. Do you think you have any nodes/swellings on your hands? Yes ☐ No ☐

If Yes, for each hand please circle the finger joint(s) where you have these nodes. (You may circle several joints).



Section 4 – Nodal status (continued)

78b. If Yes, at what age did the nodes first develop? ☐☐

78c. How many nodes do you have on the: left hand ☐☐

right hand ☐☐

79. Which hand do you write with? Left ☐

Right ☐

80a. Have you suffered from pain in the fingers for most days for at least one month?

Yes ☐ No ☐

80b. If Yes, at what age did you first develop 'significant' pain in your fingers? ☐☐

81. Do you have pain in the base of your thumb (as arrow on drawing)?

Yes ☐ No ☐

Section 5 – Family History of Osteoarthritis

82. Does/did your mother suffer from osteoarthritis of the knee/hip?

Yes ☐ No ☐ Don't know ☐

If Yes, has/did your mother had/have a total joint replacement of the knee/hip?

Yes ☐ No ☐ Don't know ☐

83. Does/did your father suffer from osteoarthritis of the knee/hip?

Yes ☐ No ☐ Don't know ☐

If Yes, has/did your father had/have a total joint replacement of the knee/hip?

Yes ☐ No ☐ Don't know ☐

84. Does/did your brothers/sisters suffer from osteoarthritis of the knee/hip?

Yes ☐ No ☐ Don't know ☐

If Yes, has/did your brothers/sisters had/have a total joint replacement of the knee/hip?

Yes ☐ No ☐ Don't know ☐

9.3 Appendix C: WOMAC Questionnare

Effects of Arginine on Osteoarthritis: A Pilot Clinical Trial

The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC)

Section 1 – WOMAC for knee

This section assesses pain, stiffness, and functional deficit **before surgery** on a scale from 0 to 4. Example:

None				Severe
0	1	2	3	4

Example of no pain	☒	☐	☐	☐	☐
Example of severe pain	☐	☐	☐	☐	☒

1. Referring to your **knees** only how much **pain** do you experience when

	None 0	1	2	3	Severe 4
a. Walking on a flat surface	☐	☐	☐	☐	☐
b. Going up and down stairs	☐	☐	☐	☐	☐
c. At night while in bed	☐	☐	☐	☐	☐
d. Sitting or lying	☐	☐	☐	☐	☐
e. Standing upright	☐	☐	☐	☐	☐

2. Referring to your **knees** only how much **stiffness** do you experience

	None 0	1	2	3	Severe 4
a. After first awakening	☐	☐	☐	☐	☐
b. Later in the day	☐	☐	☐	☐	☐

Section 1 – WOMAC for knee (continued)

3. Referring to your **knees** only how much **functional deficit** do you experience when

	None 0	1	2	3	Severe 4
a. Descending stairs	☐	☐	☐	☐	☐
b. Ascending stairs	☐	☐	☐	☐	☐
c. Rising from bed	☐	☐	☐	☐	☐
d. Rising from sitting	☐	☐	☐	☐	☐

e. Putting on socks	☐	☐	☐	☐	☐
f. Taking off socks	☐	☐	☐	☐	☐
g. Bending to the floor	☐	☐	☐	☐	☐
h. Lying in bed	☐	☐	☐	☐	☐
i. Walking on flat surface	☐	☐	☐	☐	☐
j. Getting in/out of the bath	☐	☐	☐	☐	☐
k. Standing	☐	☐	☐	☐	☐
l. Sitting	☐	☐	☐	☐	☐
m. Getting in/out of the car	☐	☐	☐	☐	☐
n. Getting on/off the toilet	☐	☐	☐	☐	☐
o. Heavy domestic chores	☐	☐	☐	☐	☐
p. Light domestic chores	☐	☐	☐	☐	☐
q. Shopping	☐	☐	☐	☐	☐

9.4 Appendix D: SF-36 Questionnaire

Effects of Arginine on Osteoarthritis: A Pilot Clinical Trial

Medical Outcomes Study Questionnaire Short Form 36 Health Survey (SF-36)

Study ID:	Date:

SF- 36 Survey: The SF-36 survey is one of many outcomes assessments designed by the Medical Outcomes Trust in Boston, MA. It is designed to approximate the improvement in health status from a medical intervention.

INSTRUCTIONS: This survey asks for views about your health. This information will help keep track of how you feel and how well you are able to do your usual daily activities. Answer every question marking the answer as indicated. If you are unsure about how to answer a question, please give the best answer you can.

1. In general, would you say your health is: (Circle One)	1. Excellent 2. Very Good 3. Good **4.** Fair **5.** Poor

2. Compared to one year ago, how would you rate your health in general at this time? (Circle One)	1. Much better now than one year ago 2. Somewhat better now than one year ago 3. About the same as one year ago **4.** Somewhat worse that one year ago **5.** Much worse now than one year ago

3. The following items are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?
(Circle the appropriate number for each question)

Activities	Yes, limited a lot	Yes, limited a little	No, not limited
a. Vigorous activities, such as running, lifting heavy objects, or participation in strenuous sports	1	2	3
b. Moderate activities, such as moving a table, Vacuuming, bowling or golfing	1	2	3
c. Lifting or carrying groceries	1	2	3
d. Climbing several flights of stairs	1	2	3
e. Climbing one flight of stairs	1	2	3
f. Bending, kneeling, or stooping	1	2	3
g. Walking more than a mile	1	2	3
h. Walking several blocks	1	2	3

i. Walking one block	1	2	3
j. Bathing or dressing yourself	1	2	3

4. During the past 4 weeks, have you had any of the following problems with your work or other regular activities as a result of your physical health? (Circle the appropriate number for each question)		
a. Cut down on the amount of time you spent on work or other activities	Yes = 1	No = 2
b. Accomplished less than you would like	Yes = 1	No = 2
c. Were limited in the kind of work or other activities	Yes = 1	No = 2
d. Had difficulty performing the work or other activities (For example – requiring an extra effort)	Yes = 1	No = 2

5. During the past four weeks, have you had any of the following problems with your work or other regular daily activities as result of any emotional problems (such as feeling depressed or anxious)? (Circle the appropriate number for each question)		
a. Cut down on the amount of time you spent on work or other activities	Yes = 1	No = 2
b. Accomplished less than you would like	Yes = 1	No = 2
c. Didn't do work or other activities as carefully as usual	Yes = 1	No = 2

6. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors or groups? (Circle one)	1. Not at all 2. Slightly 3. Moderately 4. Quite a bit 5. Extremely

7. How much bodily pain have you had during the past 4 weeks? (Circle one)	1. None 2. Very mild 3. Mild 4. Moderate 5. Severe 6. Very severe

8. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)? (Circle one)	1. Not at all 2. Slightly 3. Moderately 4. Quite a bit 5. Extremely

9. These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past 4 weeks: (Circle one number on each line)

	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	None of the time
a. Did you feel full of pep?	1	2	3	4	5	6
b. Have you been a very nervous person?	1	2	3	4	5	6

c. Have you felt so down in the dumps that nothing could cheer you up?	1	2	3	4	5	6
d. Have you felt calm and peaceful?	1	2	3	4	5	6
e. Did you have a lot of energy?	1	2	3	4	5	6
f. Have you felt downhearted and blue?	1	2	3	4	5	6
g. Did you feel worn out?	1	2	3	4	5	6
h. Have you been a happy person?	1	2	3	4	5	6
i. Did you feel tired?	1	2	3	4	5	6

10. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting friends, relatives etc.)?(Circle one)	1. All of the time 2. Most of the time 3. Some of the time 4. A little of the time 5. None of the time

11. How TRUE or FALSE is each of the following statements to you?(Circle one for each line).					
	Definitely True	Mostly True	Don't Know	Mostly False	Definitely False
a. I seem to get sick easier than other people	1	2	3	4	5
b. I am as healthy as anybody I know	1	2	3	4	5
c. I expect my health to get worse	1	2	3	4	5
d. My health is excellent	1	2	3	4	5