ATTENUATION OF MUSCLE ATROPHY THROUGH AMINO ACID SUPPLEMENTATION IN PATIENTS FOLLOWING TOTAL KNEE ARTHROPLASTY

by

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A THESIS

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Title: Attenuation of Muscle Atrophy through Amino Acid Supplementation in Patients following Total Knee Arthroplasty

Approved: _____

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Background. Total knee arthroplasty (TKA) is becoming increasingly common in the United States. Over 3.4 million older US adults are predicted to undergo primary TKA annually by the year 2030, and over 4.5 million Americans already live with a primary knee prosthesis. Immediately following surgery, significant muscle atrophy occurs, compromising strength and functional mobility of the patient. Essential amino acid supplementation has been proven to mitigate post-operative muscle atrophy in TKA patients at the level of whole muscle, and this strategy has the potential to attenuate individual muscle fiber atrophy as well. ***Methods.*** Data collection for this Honors Thesis is part of an ongoing double-blind, placebo-controlled randomized clinical trial. This Honors Thesis will present blinded raw data from 14 patients who have completed the study. Patients are randomized to ingest either 20 g of EAAs or placebo twice daily between meals for 1 week before and for 6 weeks after TKA. At baseline, 2 and 6 weeks post-TKA, an MRI was performed on each leg to measure muscle mass over time. In addition, each subject had bi-lateral biopsies performed in the operating room just prior to surgery and again at either 1 or 2 weeks post-TKA

(random allocation within each cohort). Digital analysis methods were developed to quantify muscle volume (MRI) and muscle cell cross-sectional area (histology) . ***Results***. This blinded study is not yet complete and therefore this Honors Thesis will not report any results. The data reported are coded and randomized in order to maintain the integrity of the clinical trial. ***Conclusion.*** Unfortunately, the effectiveness of essential amino acid supplementation in attenuating muscle atrophy cannot be assessed in this Honors Thesis. However, the precision of the analysis techniques developed here can be evaluated and confirmed. Furthermore, the clinical importance of EAA supplementation in potentially decreasing post-operative muscle atrophy in TKA patients is substantiated as this surgery is becoming increasingly prominent in the US.

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Introduction

Total knee arthroplasty (TKA), or total knee replacement, is defined as the surgical reconstruction of the knee joint with the ultimate intention of restoring physical integrity and function to the damaged or degenerated joint. Total knee replacements are becoming increasingly common in the United States, with over 4.5 million Americans currently living with a primary total knee prosthesis[1]. Over 650,000 total knee replacements were conducted in 2010[1], and by 2030 it is projected that nearly 3.4 million primary TKAs will take place in the United States annually[2]. Older individuals most often receive this treatment, with over 98% of TKA surgeries involving patients over the age of 45[1].

The primary diagnosis leading up to TKA is osteoarthritis of the knee. This involves chronic inflammation, and progressive deterioration, of hyaline cartilage, eventually leading to bond-on-bone contact within the knee joint. This condition is painfully debilitating and severely limits movement about the knee joint, contributing to immobility and sedentarism in the affected patient. The adoption of an inactive lifestyle in response to this chronic injury greatly increases risk of mortality through increasing risk of developing cardiovascular and musculoskeletal pathologies, as well as metabolic syndrome[3]. Therefore, the implications of knee osteoarthritis should be considered serious and life threatening to those effected by it.

A comprehensive solution is necessary to remedy the chronic pain associated with knee osteoarthritis in order to bolster active lifestyles and improve quality of life for the affected older population. Total knee arthroplasty is a remarkably successful treatment solution for patients suffering from chronic osteoarthritic knee pain. Over

95% of TKA patients (96% of whom were suffering from osteoarthritis) reported satisfaction with their surgical outcome and 90% reported dramatic pain relief as a result of surgery[1]. Increases in strength in the operative leg improve knee functionality after surgery[4] and drastically improve overall quality of life for patients. However, surgery alone is insufficient in returning TKA patients to their preoperative levels of strength and function, even though their chronic knee pain is often remedied. Many patients experience significant quadriceps muscle atrophy post-operatively, ultimately compromising the ability to maximally regain knee strength and function to levels similar to healthy age-matched controls[5,4,9,33]. Muscle atrophy following TKA occurs at a rate of 1% per day for the first 2 weeks after surgery[9]. Long-term muscle atrophy limits rehabilitation potential and diminishes complete recovery. Further complicating the situation, the older adult demographic receiving TKA inevitably experiences sarcopenia, or age-correlated muscle degeneration, in addition to post-operative muscle atrophy. Sarcopenia is experienced by almost half of people over the age of 80, and contributes largely to age related disability in general[6]. TKA exacerbates sarcopenia and accelerates total muscle loss in patients after surgery. In conjunction with one another, these atrophic factors greatly impede the ability of TKA patients to return to baseline levels of strength and function after surgery. Unfortunately, the atrophy in this context is likely permanent due to the age-associated muscle dysfunction inherent in sarcopenia[15].

There is therefore a present and increasing demand for improving post-operative recovery for TKA patients in order to attenuate the muscle atrophy associated with the rehabilitation process. Decreasing overall muscle loss post-operatively translates into

shorter and easier recovery periods for patients, as well as a better end-result in regards to strength and function of the joint. It is known that essential amino acid (EAA) supplementation increases lean muscle mass[7,8], and this has the potential to remedy the negative muscle atrophy experienced by patients undergoing a total knee replacement. In a 2013 study, Dreyer *et al*. found that twice daily ingestion of 20 grams of EAAs between meals for 1 week before TKA and 2 weeks-post helped reduce the degree of post-operative muscle atrophy and associated weakness in patients undergoing total knee arthroplasty[9]. These findings have far reaching clinical implications not only in the realm of total knee replacements, but in joint prosthesis in general. If essential amino acid supplementation can successfully remedy muscle loss post-operatively, simple administration of them before and after surgery could help patients with TKAs or other prostheses recover more quickly and completely after surgery.

A 2013 study published by Dreyer *et al*, showed that essential amino acid supplementation was successful in attenuating quadriceps and hamstrings/adductor muscle atrophy in TKA patients following surgery[9]. However, Dreyer and colleagues did not address how muscle fiber cross-sectional area was affected by EAA supplementation during recovery. Therefore, the primary purpose of this study is to assess how twice daily ingestion of 20 grams of EAAs for 1 week before and 6 weeks after TKA influences changes in muscle cell cross-sectional area. Secondarily, this study seeks to confirm their previous findings (Dreyer et al, 2013) that EAA supplementation attenuates muscle loss and accelerates the return of functional mobility at 6 weeks post-TKA. By coupling muscle cell cross-sectional area data from histological analysis with muscle volume data from MRI scans, this Honors Thesis aims

to assess the effectiveness of EAAs in deterring muscle atrophy at both the whole-muscle and cellular level. This bilateral approach aims to address how individual muscle cell alterations ultimately affect whole muscle structure and function after TKA. It is hypothesized that twice daily ingestion of 20 grams of essential amino acids will decrease muscle atrophy (both volume and cross-sectional area) of the quadriceps muscle group in both the operative and non-operative leg following total knee arthroplasty.

Background

Osteoarthritis

Chronic osteoarthritis (OA) is a crippling condition in which joint articular cartilage and its underlying bone become progressively degenerated, resulting in pain and stiffness during movement. OA is currently the most common reason for hospitalization for adults aged 45 to 85 in the United States, with a 160% increase in admission rates between 1997 and 2011[10]. Prevalence of osteoarthritis will increase drastically by 2030, with over 67 million adults (totaling 25% of the adult population) predicted to experience the debilitating symptoms[11]. In terms of pathologic origin, osteoarthritis results from a failure in the joint's repair process whereby damaged cartilage fails to be replaced with new and healthy cartilage as a function of biomechanical and biochemical alterations in the joint space[12]. Ultimately, an imbalance develops favoring degradation of chondrocytes and the extracellular matrix, producing a vulnerable joint environment unable to withstand continued mechanical stress. Being avascular, cartilage is largely restricted from blood circulation, and thus essential nutrients and oxygen availability in the event of an injury. Cartilage is also aneural, meaning that these degenerative joint changes fail to produce clinical symptoms until adjacent innervated tissues, such as bone, become damaged[12]. Consequentially, OA is often diagnosed late and patients can sustain severe cartilage diminution before intervention is enacted.

OA of the knee is the diagnosis in over 96% of TKA cases, making it the principle diagnosis associated with this surgery[1]. Knee osteoarthritis, specifically, is characterized by progressive deterioration of the hyaline articular cartilage and bone of

the knee, accompanied by ligament and tendon alterations, resulting in inflammation of the joint space and painful, constrained ambulation[13]. This cartilage loss eventually progresses into bone-on-bone contact between the distal femur and proximal tibia, resulting in intense pain during articulation. TKA is most often prescribed to remedy this pathology, and it is remarkably effective in reducing pain caused by OA by directly removing the source. However, many confounding factors complicate post-operative recovery and limit a patient's ability to return to their pre-operative levels of knee strength and function.

Sarcopenia

The generally older population experiencing OA and the associated pain often suffers from skeletal muscle deficiencies due to sarcopenia. Sarcopenia is defined as the progressive and involuntary loss of skeletal muscle mass as a function of normal aging. It is a phenomenon that contributes to development of frailty in old age,[14] and is directly related to increased risk of falls, development of disability, and loss of independence[15]. In the year 2000, it is estimated that the United States spent $18.5 billion on healthcare directly attributable to sarcopenia, accounting for 1.5% of total healthcare costs that year[16]. After the age of 35, 3-8% of total skeletal muscle mass may be lost per decade due to sarcopenia, and this loss is accelerated after 60 years of age[17,18]. Muscle power output simultaneously decreases with age[19] and likely accounts for the observed discrepancies in the elderly relating to functional independence[20,21]. The quality of skeletal muscle components also declines with age, negatively influencing cross-bridge cycling and excitation-contraction coupling, further exacerbating functional declines in older individuals[17].

The etiology of sarcopenia remains unknown, although it is likely caused by a varying combination of possible cellular mechanisms. These include declines in conduction velocity of motor neurons (specifically type II), motor unit loss, muscle cell loss, interference in excitation-contraction coupling, mitochondrial DNA deletions, alterations in satellite cell physiology, hormonal changes, impaired tissue response to nutrients, malnutrition, all of which are exacerbated by a lack of physical activity[15]. Regardless of the individual involvement of each of these contributing factors, it remains that the muscle fiber atrophy associated with sarcopenia is due to an imbalance between muscle protein synthesis and breakdown. There is evidence to suggest that the muscle protein synthesis pathway in sarcopenic adults is not disrupted due to a lack of difference in anabolic response between healthy young and old adults in response to a protein (essential amino acid) stimulus[15]. Therefore, the catabolic pathway seems to dominate protein turnover in older adults experiencing sarcopenia, resulting in a net decrease of muscle protein content and explaining the diminished muscle mass and force production.

The atrophy associated with sarcopenia also has a dramatic influence on bone health. The connection between osteoarthritis and sarcopenia serves to manifest the important relationship between skeletal muscle mass and bone strength in the elderly. The application of mechanical force on bone increases bone modeling and remodeling, processes which ultimately increase bone mass and strength[22]. Considering that the greatest amount of force applied to bones is from skeletal muscle[22], it is clear that voluntary contraction of muscle contributes to bone mass and strength. As sarcopenia progresses and muscle mass diminishes, the ability of muscle to provide adequate force

on bone for remodeling declines, and accordingly, bone health suffers. Thus, muscle loss is accompanied by bone loss. In fact, quadriceps weakness (15%-18% below baseline) is associated with development of osteoarthritis in a number of studies[23,24]. This highlights the importance of muscle mass retention on overall health and mobility, especially in the elderly.

The Knee Joint and the Quadriceps Muscle Group: Functional Stability

The knee joint is the largest and most superficial joint in the human body. Although well constructed, this synovial joint is considered relatively weak due to the variance in shape of its articular surfaces, as well as its requirement of adequate muscle mass for proper strength and function[25]. The quadriceps femoris muscle group performs extension of the knee and stabilizes it during ambulation by serving as one of the most powerful muscle groups in the human body. The quadriceps group is comprised of 4 muscles, the rectus femoris, vastus lateralis, vastus intermedius, and vastus medialis. Common practical function of these muscles includes walking, running, jumping, rising from sitting or squatting, or climbing stairs. Considering many elderly people, and especially those with osteoarthritis[11], experience increased difficulty performing these activities with age, it is clear that maintaining specifically quadriceps function through muscle mass retention is of primary concern in keeping the elderly dynamically stable[26]. This becomes even more important in the context of an elderly patient undergoing a total knee arthroplasty.

A severe decline in specifically quadriceps muscle mass and strength has been observed for TKA patients during the post-operative recovery process[9,27], and this is highly correlated with declines in functional mobility more so than other measures of

physicality[34]. Dreyer and colleagues observed an 18% reduction in quadriceps muscle mass in control TKA subjects 6 weeks after surgery[9]. Considering the patient population, the combined degenerative effects of sarcopenia and low mobility seem to have combined negative affects on quadriceps muscle mass and appropriately affect functional mobility post-operatively. Quadriceps weakness can also encourage further injury in the patient. Weakness in the operative leg causes changes in movement patterns that result in increased loading of the non-operative leg, exacerbating potential osteoarthritic damage in the non-operative leg and risking necessity for contralateral knee replacement in the future[28].

Mobility Reduction and Muscle Atrophy

Postoperative mobility impairments directly challenge TKA patients' efforts to return to their preoperative levels of knee strength and function. While not rendered completely immobile, TKA patients remain severely limited in activity after surgery; despite twice-daily physical therapy sessions that begin postoperative day one. Extensive loss of muscle mass, strength, and function as a product of prolonged hospitalization and reduced mobility likely contribute to a longer recovery times for patients after surgery[29]. Models of disuse atrophy in human subjects indicate a decrease in muscle protein synthesis of approximately 60%, as well as a reduction in the anabolic stimulus of EAAs by 50%, after four weeks of sustained immobility[30]. This decrease in muscle protein synthesis results in a loss of 0.3% - 0.8% muscle cross-sectional area per day[30,31]. This loss in muscle mass associated with surgery and recovery is likely to accelerate sarcopenia in elderly patients[9], ultimately extending recovery times and limiting positive clinical outcomes. The atrophy experienced by older patients during

this period is likely permanent[15], which severely compromises long-term functional gains[32]. In addition, immobility-derived muscle loss sustained preoperatively due to chronic knee pain further challenges recovery efforts by decreasing baseline skeletal muscle mass. This likely occurs because of the time delay between the onset of osteoarthritic symptoms and surgical intervention, which may require years to achieve. Thus, it follows that patients suffering from chronic knee osteoarthritis have about two-thirds the quadriceps muscle mass of comparative adults[33]. It is clear that patients undergoing a total knee replacement are functionally challenged post-operatively, and attenuation of atrophy in the quadriceps muscle group is directly correlated to improvements in strength and function, ultimately translating to shorter recovery times and better overall outcomes for patients.

Attenuating Muscle Atrophy

In general, administering hormones, performing exercise, and manipulating nutrition are all potential strategies in attenuating loss of muscle mass and function[14]. Hormonal therapy such as the use of testosterone or epinephrine/cortisol blockers has positive effects on muscle loss and function, however, treatment involving them is often accompanied by undesirable and unpredictable complications. Therefore, the use of hormones in this clinical context is limited[14]. Exercise increases functional mobility in elderly patients experiencing sarcopenia, however, it does not seem to actually attenuate loss of muscle mass[14]. However, some studies indicate early onset of exercise post-operatively aids in recovery of functionality and provides a "higher plateau" for improvement, translating to more independence and mobility[34]. However, the elderly population primarily receiving TKA is largely limited in their ability to perform

resistance exercise as a function of osteoarthritic pain and compromised muscle mass from sarcopenia. Therefore, this strategy, too, is limited in the cohort of subjects under investigation. Although hormonal therapy and exercise seem to be ineffective strategies, manipulation of nutrition has vast potential for remedying loss of muscle mass and function.

It is known that high-protein diets increase protein synthesis based on expansion of amino acid availability[35], and this phenomenon happens dose-dependently[36], meaning that higher rates of protein intake correspond to higher rates of protein synthesis. Ingestion of dietary protein above DRI values is known to stimulate the fractional synthesis rate (FSR) of muscle protein during protein turnover[37], and this is positively correlated with strength increases[38]. It is likely that higher muscle protein turnover rate results in formation of new myofibrillar proteins that ultimately function with greater capacity[14]. Essential amino acid supplementation increases muscle mass, strength and function similarly to a high protein diet, independent of exercise[39,40]. In a recent study, elderly individuals who daily ingested amino acids above normal dietary recommendations for 3 months had improved muscular strength and function[41]. In addition, it is likely that amino acid supplementation also increases skeletal muscle metabolic function by increasing mitochondrial protein synthesis, ultimately allowing for greater oxidative efficiency[42]. These findings have far reaching clinical implications. If simple oral supplementation of amino acids improves muscle strength and function, simple administration of them could help attenuate muscle loss both from sarcopenia and the impaired mobility associated with hospitalization and post-operative recovery. Findings from the Dreyer Lab provide proof-of-principal for the potential of essential

amino acid supplementation to attenuate muscle loss post-operatively in older adults having TKA[9], providing inspiration for this study.

Nutrition and muscle protein synthesis seem to be negatively affected by the narcotics often prescribed to patients after surgery to manage pain and encourage range of motion in the operative knee. Hydrocodone/acetaminophen, oxycodone, and dilaudid (hydromorphone) are common opioid narcotics prescribed to patients after TKA to manage pain. In a recent review, Wiffen *et al.* found that anorexia or decreased appetite was a side affect of opioid use in 1 out of 8 prescribed patients[43]. Partial or complete loss of appetite could result in a decrease in dietary protein ingestion and the absence of the associated anabolic stimulus. This could ultimately encourage muscle atrophy if muscle catabolic processes remain active. Dreyer *et al.* observed a decrease in protein ingestion in control subjects 2 weeks after TKA, and they surmised that this dietary deficiency could be a contributing factor to postoperative muscle atrophy and observed declines in strength and function[9]. It is possible that narcotic use following surgery could contribute to this decrease in dietary protein ingestion through appetite suppression.

Methods

Subjects

The subject pool for this experiment consisted of adult males and females between the ages of 50 and 80 who were scheduled to undergo primary total knee arthroplasty (TKA). The study aimed to enroll 40 subjects. For the analysis presented here, 14 of the 40 subjects had completed the experimental protocol. Through collaboration with Slocum Center for Orthopedics and Sports Medicine in Eugene, Oregon, subjects were chosen from a pool of potential candidates scheduled to receive a primary knee joint prosthesis. Candidates were cleared prior to inclusion into the study for various preexisting medical conditions, such as significant heart, kidney, liver, or blood disease, untreated endocrine disease, peripheral vascular disease, active cancer, or past treatment with anabolic steroids or oral corticosteroids (>1 week). Patients undergoing a revision total knee arthroplasty were also excluded due to possible bias in recovery process expectations. All subjects completed a detailed informed consent agreement before participating in the study. Upon enrollment, each subject was assigned to either a treatment (EAA) or placebo group (NEAA) using a set of random assignment procedures designed by the Principal Investigator. Considering the double-blind nature of this study, all researchers were unaware of the group-identity of the subject, and all subjects remained unaware of their placement within the groups.

Trial Design

This placebo-controlled, double-blinded study began in January of 2015 with Dr. Hans Dreyer as Principal Investigator. The study incorporates a dual armed, parallel-designed trial with a group allocation ratio of 1:1 between treatment and placebo groups. All subjects completed MRI imaging at 2 weeks pre-TKA (baseline), 2 weeks post-TKA, and 6 weeks post-TKA for the purpose of analyzing quadriceps muscle volume. Muscle biopsies were obtained on the morning of the TKA operation, and at either 1 week post-TKA or 2 weeks post-TKA (randomized), for the purpose of analyzing muscle fiber cross-sectional area. Baseline measures of quadriceps volume and muscle cross-sectional area were obtained prior to the total knee replacement, as well as before the introduction of amino acid treatment (MRI only).

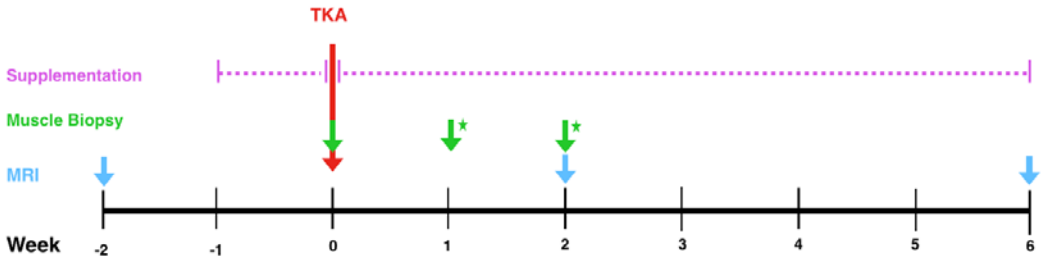


Figure 1: Trial design timeline

This timeline illustrates the trial design and time points of data acquisition. Arrows in blue represent MR image acquisition; Arrows in green indicate muscle biopsy acquisition (* denotes second biopsy – each subject contributed one biopsy at either randomly selected time). Arrow in red indicates day of TKA operation. Dotted line in pink represents duration of amino acid supplementation (twice daily, 49 days total).

Supplementation

Subjects in the treatment group were orally administered a regime of 20 grams essential amino acids (EAA) twice-daily for 1 week before, and 6 weeks after TKA. The placebo group was twice- daily given 20 grams nonessential amino acids (NEAA) for the same duration. Supplementation began one week prior to the TKA operation, resumed again one day post-surgery, and continued 42 additional days, for a total of 49 days of supplementation. Ingestion of the amino acid supplement occurred at 10 am and 2 pm daily. After surgery and during inpatient hospitalization, subjects ingested their morning supplement one hour after their early physical therapy session. Subjects ingested their second daily supplement an hour after completion of their afternoon physical therapy session. Subjects completed three physical therapy sessions per week for the first two weeks after TKA intervention. Supplement ingestion occurred after regularly scheduled physical therapy sessions in order to maximize the anabolic effects of the essential amino acids in muscle protein synthesis[44,45]. Subjects were encouraged to continue this behavior after inpatient hospitalization in outpatient rehabilitation. The composition of the essential amino acid supplement was: histidine, 2.2 g (11% of total); isoleucine, 2.0 g (10%); leucine, 3.6 g (18%); lysine, 3.2 g (16%); methionine, 0.6 g (3%); phenylalanine, 3.2 g (16%); threonine, 2.8 g (14%); and valine, 2.4 g (12%). Subjects in the placebo group were administered a supplement of 20 g (100%) alanine (non-essential amino acid). The EAA concoction was mixed offsite at Northwest Compounders in Tualatin, Oregon. A study coordinator distributed either the EAA or NEAA supplements, which were coded to ensure blindedness of researcher and study

subject. Records of supplementation were maintained throughout the study and were routinely monitored by research personnel during regular visits.

Total Knee Arthroplasty

On the morning of the operation, each subject was admitted to Sacred Heart Medical Center at RiverBend (Springfield, Oregon) in a postabsorptive state. Either an epidural, spinal, or general anesthesia was administered. Intravenous Propofol was used to initiate general anesthesia, and either inhalational desflurane or sevoflurane (sometimes combined with a muscle relaxant, rocuronium bromide) was used to maintain anesthesia throughout the surgery. A 10-cm wide Zimmer inflatable tourniquet was placed on the proximal third of the operative thigh and inflated to 300 mmHg in order to minimize perfusion during surgery. The tourniquet was removed after the major components of the surgery were completed to allow reperfusion of the operative limb.

MRI Acquisition

All magnetic resonance imaging for the study was conducted at the Lewis Center for Neuroimaging (LCNI) at the University of Oregon in Eugene. Patients arrived at LCNI two weeks prior to their scheduled TKA surgery for baseline MRI scans of the bilateral lower extremities between the anterior iliac spine of the pelvis and the tibial plateau. A Siemens Magnetom Skyra T3 system was used to acquire 90-2mm thick transverse slice images using the following scan parameters: T1 weighted; TR: 600 ms; TE: 9.9 ms; echo spacing 9.86 ms; 2D Distortion Correction and Prescan Normalize filters; minimum flip angle 150°. Automatic table repositioning was used

during the three employed 90-slice scans to grant contiguousness between slices. The subject was positioned supine within the scanner and two flexible Siemens Body 18 coils (18 channel, phased arrayed coils) were placed and adjoined anteriorly over the subject's pelvis and distal femur to acquire images in the area of interest. A Siemens Spine 32 array coil was used in conjunction with the Body array coils, and was located posteriorly underneath the subject during the scans. Early in the study, placement of the flexible Body coils directly on the subject resulted in anteromedial visual interference in the image. This interference greatly complicated data analysis. By including an additional layer of foam (appx. 1.5 cm), the interference was dampened and the issue was resolved. The Dixon technique was applied to obtain additional "water" and "fat" images, which served useful in the analysis protocol. Patients returned to LCNI for additional MRI acquisition two weeks and six weeks post-TKA, and the same scan parameters and equipment were employed. Images for the study were made available to the Principal Investigator, who then concealed the subject identity and time point of acquisition before releasing each set of scans for analysis.

MRI Analysis, Determination of Quadriceps Volume

Each of the subject's three image subsets (2w pre-op, 2w post-op, 6w post-op) was encoded by the Principal Investigator to ensure blindedness during analysis. The Principal Investigator produced a single 9-image sequence (referred to as a stack) from each subset; each stack consisted of three sub-sequences, each of which was applied with a different image filter – "muscle", "water", or "fat". Each of the three sub-sequences within the 9-image stack was comprised of three images (proximal, middle, distal thigh) from the area of interest. Each sub-sequence consisted of the same three

images, albeit under different filters. Images (1-3) consisted of a proximal, middle, and distal image under the “fat” sub-sequence filter. Images (4-6) consisted of the same three images under the “muscle” sub-sequence filter. Similarly, Images (7-9) were the identical images under the “water” sub-sequence filter. Images were chosen from the mid-thigh region (~5 cm window) based on clarity and lack of distortion/interference. Figure 2 shows an example of a single image stack utilized for analysis.

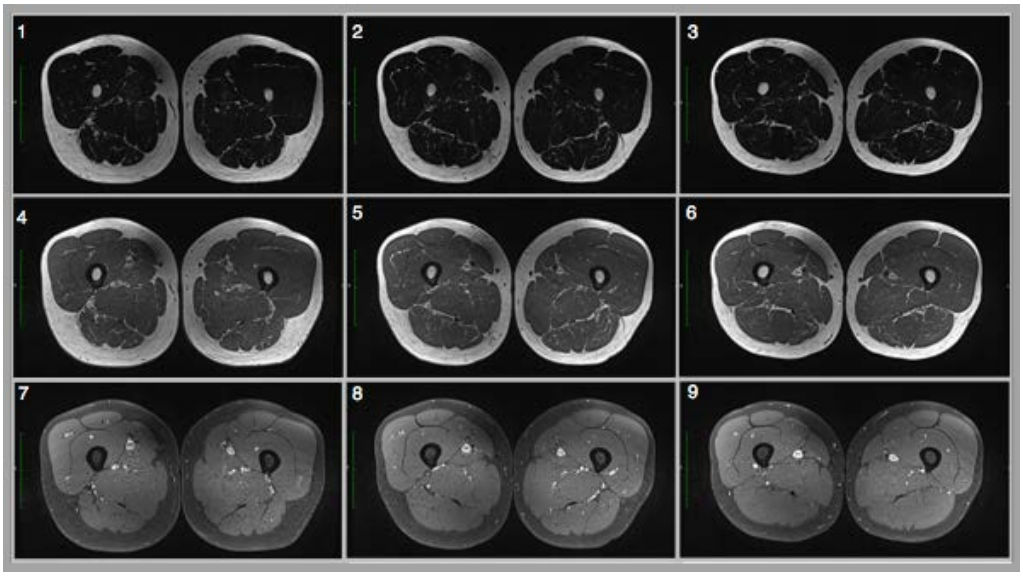


Figure 2: A single image stack for a subject.

Images [1,2,3] represent a “fat” sub-sequence; Images [4,5,6] represent a “muscle” sub-sequence; Images [7,8,9] represent a “water” sub-sequence. Images [1,4,7] are identical proximal thigh images; Images [2,5,8] are identical middle thigh images; Images [3,6,9] are identical distal thigh images.

MR image analysis was conducted using ImageJ, a dynamic Java-based computer application developed by the National Institutes of Health (NIH). A single 9-image stack (Figure 2) was imported into ImageJ in RGB (red, green, blue) format. While all three image sub-sequences were available during analysis, only the “fat” sub-sequence (Images [1,2,3]) was used to quantify area of muscle because it manifested

contrasts between muscle and fat most accurately (Figure 3, Image 1). Beginning at Image 1, the Wand tool, which uses a tolerance gradient (1-100) for selection of similar particles based on color homogeneity, was used (tolerance = 25) to highlight the femur and quantify its area in the proximal, middle and distal image (Figure 3, Image 2). Area of the femur for each image was then recorded. The Wand tool (tolerance = 25) was used again to select and remove subcutaneous fat from the cross-sectional image of the thigh. Left with exclusively the skeletal muscle of the thigh (Figure 3, Image 3) the quadriceps muscle group was separated from the hamstrings muscle group by tracing both the medial and lateral intermuscular septums, which divide the anterior and posterior compartments of the thigh (Figure 3, Image 4). This manual delineation was verified for accuracy by cross-referencing the tracing with the identical images in other sub-sequences ("muscle" and "water"). This "raw" area of the isolated quadriceps was measured in the proximal, middle, and distal Images (1-3) and was recorded. Next, intramuscular fat was isolated from skeletal muscle by using a thresholding procedure to select and quantify fat within the muscle based on a range of particle shades. Prior to thresholding, the image stack was converted from RGB format to 8-bit, or binary, format. In addition, the medullary cavity of the femur, which appears light grey in the Image, was selected and darkened in Images (1-3) order to ensure its area was not included in quantification of intramuscular fat. Manual thresholding using the "Otsu" method was chosen because it minimizes intra-class variance within each of the two tissue types (muscle and fat) and maximizes inter-class variance between them[46]. Beginning at Image 1, the upper bound of the selection module was set to near maximum (254/255) (maximum would include the white background in the selection)

in order to include the lightest possible shades in the threshold window. The lower bound was then manually manipulated to include as much intramuscular fat as possible in the threshold window (selected particles appear red) without including skeletal muscle (Figure 3, Image 5). The area of these selected particles was then measured and recorded. This procedure was repeated for Images 2 and 3. Quadriceps muscle area was calculated by subtracting the raw quadriceps area (Figure 3, Image 4) from the combined area of the femur and intramuscular fat for each image.

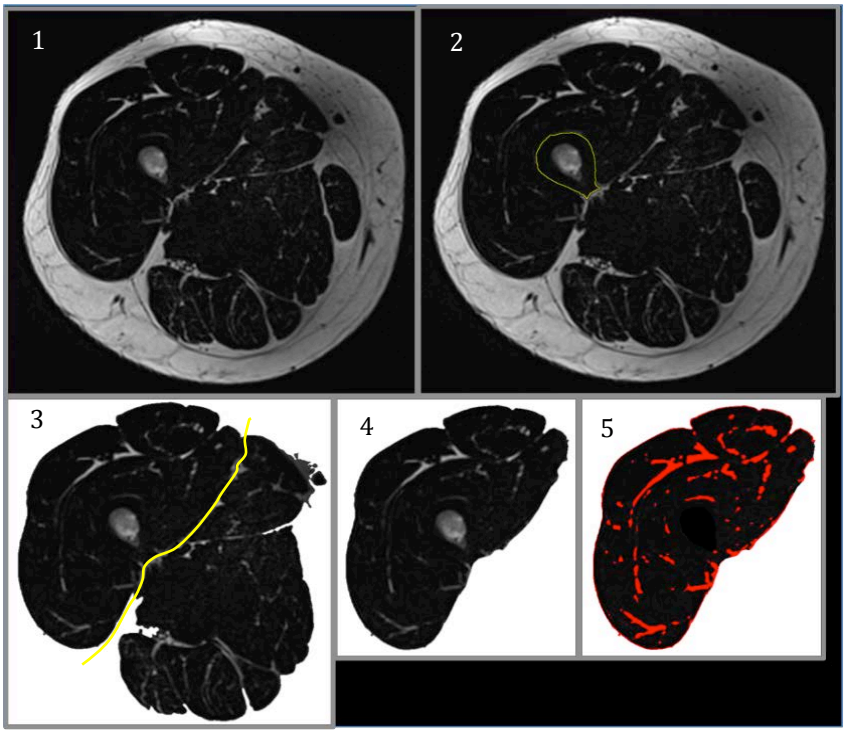


Figure 3: Image series detailing muscle volume determination from an MRI stack

[1] displays an unedited MRI image of the "fat" subsequence. [2] shows the femur highlighted in the image. [3] shows the skeletal muscle of the thigh, with subcutaneous fat removed. [4] shows the quadriceps muscle, separated from the hamstrings by tracing the intermuscular septums. [5] shows the intramuscular fat within in the quadriceps, highlighted in red using the thresholding tool.

Prior to calculating quadriceps volume from the area measurements described above, each image set was assigned a scale for converting area measurements from

pixels^2 to cm^2. Because the view size of the image file often varied, each stack received its own scale. The green scaling bar denoting 10 cm (located on the left aspect of each image, see Figure 2) was measured within ImageJ in order to quantify the equivalent measurement in pixels. This ratio of (pixels/cm) was squared in order to get a (pixels^2 /cm^2) ratio, which each area measurement was divided by in order to convert area measurements from pixels to cm.

Quadriceps muscle volume for a subject was calculated by multiplying the average quadriceps area from the proximal, middle, and distal image of each subset (2w pre-op, 2w post-op, 6w post-op) by the total distance between the proximal and distal image. Counting the number of images located between the proximal and distal images, and multiplying by the slice thickness of 2 mm, rendered this quantity. Distance was approximately 5 cm for each stack, though it varied based on the location of each image within the subset chosen for analysis. A "distance factor" of 10% was added to each distance measurement to account for the 0.2 mm separation between slices within each subset.

Acquisition of Muscle Biopsies

Muscle biopsies were obtained from the vastus lateralis muscle of both the operative leg and non-operative leg on the morning of TKA, and at 1 or 2 weeks post-operation (randomized, see Figure 1). Each subject thus contributed four muscle biopsies for analysis. Baseline biopsy samples were obtained by the surgeon in the operating room (OR) prior to application of the tourniquet for surgery. Biopsies were obtained using a suction-modified Bergstrom biopsy needle, inserted into the muscle through a previously made 1-cm incision. Correspondents from the Dreyer Lab

received and processed the tissue in the OR immediately after acquisition. Each biopsy was mounted on a short needle inserted through a 1-cm^2 square of cork. This was then bonded with OCT (Optimal Cutting Temperature) compound to a cryostat chuck, before being frozen in isopentane cooled to the temperature of liquid nitrogen for approximately 30 seconds. Frozen samples were then transported back to the Dreyer Lab where they were labeled with the appropriate subject code and kept at -80° C. The Principal Investigator performed the second biopsy in the Dreyer Lab facility at the randomly assigned time (1 or 2 weeks post-op). Standard aseptic procedures were performed and a local anesthesia [1% Lidocaine HCl (10 mg/mL)(Hospira Inc, Lake Forest, IL)(approximately 10 cc)] was employed during the procedure. Samples were processed identically as before and kept at -80° C for future analysis.

Immunohistochemistry and Fluorescence

Prior to histological preparation, the principal investigator retrieved a single subject's four muscle biopsies (pre- and post-surgery, left and right leg) and assigned each a numerical code (1-4) to conceal the time point of acquisition. The pre-mounted samples were then placed in a Leica CM1850 UV cryostat and allowed to equilibrate to the cutting temperature of -21° C. Each of the four biopsies was cross-sectioned at 7 μm and serially collected in a single vertical row on two individual Fisherbrand Superfrost Plus microscope slides. Each biopsy contributed approximately 4-6 sections per row on both slides. Muscle sections were allowed to air-dry at room temperature for approximately 1 hour before being fixed in cold acetone (4° C) for 3 minutes. Slides were allowed to dry before a Pap Pen was used to circumscribe a hydrophobic barrier around the biopsy sections. Muscle sections were washed in PBS [(phosphate-buffered

saline)(3x3min)] before being incubated with rabbit anti-laminin primary antibody (IgG1 ((1:1000) in PBS, DAKO, Carpinteria, California)) or ((1:200) in PBS, Sigma Aldrich, St. Louis, Missouri) for 1 hour. Muscle sections were again washed in PBS (3x5 min) before being treated with 3% hydrogen peroxide (H_2O_2) in PBS for 7 minutes to block endogenous peroxidase activity and non-specific staining. Sections were again washed in PBS (3x3 min) before being incubated with goat anti-rabbit IgG1 secondary antibody (target is laminin antibody, above (AlexaFluor 488 (1:500)(Invitrogen, Carlsbad, California))) for 1 hour. Muscle sections were washed in PBS (3x3 min) before 3% NDS (normal donkey serum; Jackson Immunoresearch, West Grove, Pennsylvania) in PBS was applied for 1 hour to block additional non-specific staining. Muscle sections were again washed in PBS (3x5 min) before being applied with SlowFade Diamond anti-fade mount with DAPI (labeling all nuclei)(ThermoFisher, Eugene, Oregon). VECTASHIELD Antifade Mounting Medium (Vector, Burlingame, California) was applied and a coverslip was placed on the sample. Sections remained at 4° C overnight (~12-16 hrs) before immunofluorescent images were obtained.

Fluorescent Microscopy

Muscle tissue cross sections were visualized and photographed using a Leica DM4000B fluorescent microscope, equipped with a high-speed Leica DFC360 FX camera, a DFC 295 color camera, and a Prior Lumen 200 epifluorescence light source. The Leica Application Suite (LAS) software was used for image acquisition. Beginning with the first section in row 1 (corresponding to the first biopsy), one laminin image was obtained from the visual field using λ=488 nm excitation light. Once the image was

taken and filed for a given location, the microscope stage was moved left, completely out of the current field of view, to expose a new group of cells for image acquisition. After the right border of the muscle section was reached, the stage was returned to its starting position on the left side of the slice, below the field of view of the previously captured image. The specific location of image acquisition within the muscle slice depended largely on the quality of the tissue and laminin stain at that site. Care was taken to avoid photographing the same cells multiple times. Eight individual fields of view were photographed per biopsy, encompassing 32 total laminin images per biopsy.

Histological Analysis

Skeletal muscle cell cross sectional area (CSA) was determined using ImageJ analysis software. Laminin immunofluorescent images were used during this analysis to identify the borders of each muscle cell in the field of view (Figure 4, Image 1). Each laminin image was individually imported into ImageJ. Image brightness and contrast were then simultaneously increased in order to intensify the extracellular matrix (ECM) signature, and to reduce the appearance of any non-specific background staining (Figure 4, Image 2). The thresholding tool was then used to isolate the muscle cells (dark in the image) from the ECM (bright white). The threshold parameters were “applied,” and the image was converted to a manipulation-friendly binary image that separated individual muscle cells (white) from ECM (black)(Figure 4, Image 3). Any discrepancies in the ECM were manually amended with the Brush tool (width: 2-4) in order to fully complete the perimeter of as many cells in the window as possible. The identity of very small muscle cells was confirmed by cross-referencing the binary image with the original laminin image. Once the cellular borders in the binary image were augmented,

the image was cross-referenced with the original laminin image a final time to confirm the identity of the muscle cells as such. Confirmed muscle cells with an uninterrupted membrane lying entirely within the viewing window (not on the border) were colored a non-specific shade of grey for differentiation from damaged cells or non-muscle cells. This process was repeated until the entirety of muscle cells within the window was highlighted. The appropriate pixel-scale ratio (1.953 pixels/μm) was entered in the Scale window in order to convert area measurements from pixels to μm^2. The thresholding tool was again used to select exclusively the grey-highlighted cells, excluding damaged muscle cells, non-muscle cells and the ECM. The Analyze Particles macro was used to expeditiously measure the cross-sectional area of the individual cells in the image. "Show Outlines" and "Display Results" were selected in the drop-down menu in order to auto-generate an outline "drawing", or rendering, of the cell borders, along with area measurements for each cell (Figure 4, Image 4). The macro auto-assigned numbers to each cell, which coincided with individual area measurements listed in the Results table. Muscle cell cross-sectional area measurements were then imported into Microsoft Excel, where average fiber CSA was calculated for each biopsy. Each "drawing", or rendering, of the group of cells was saved for reference purposes.

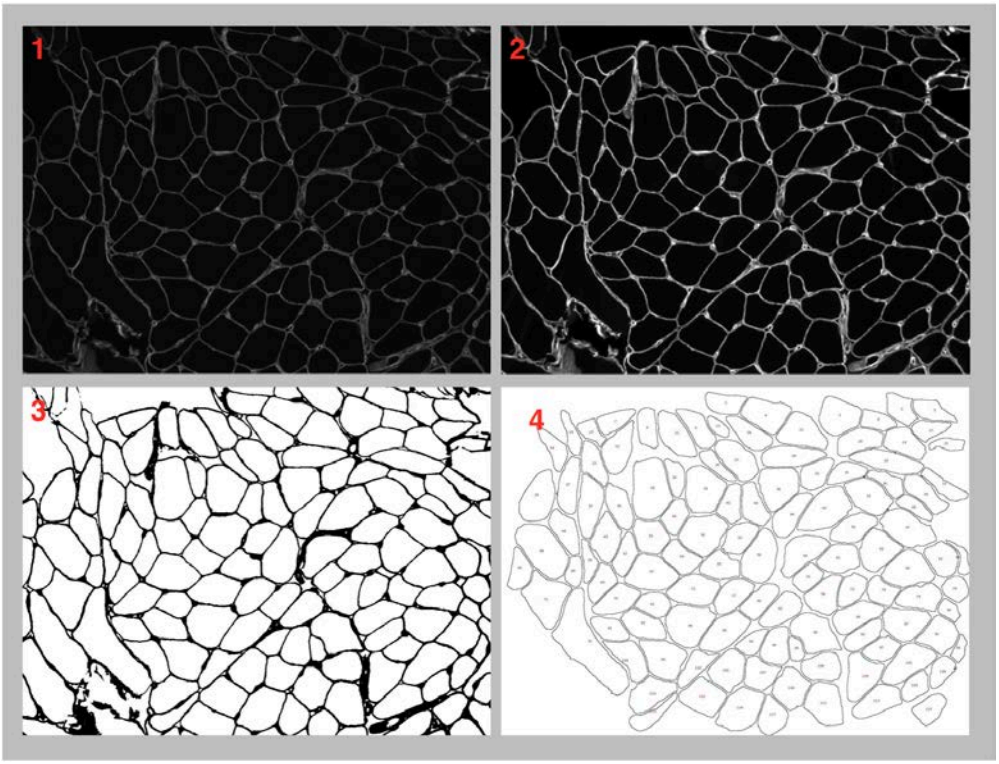


Figure 4: Image sequence detailing CSA determination from a single laminin-stained muscle biopsy image (7 μm).

[1] shows an unedited laminin image. [2] shows the image with enhanced brightness/contrast. [3] shows the image in binary form, converted using the thresholding procedure. [4] shows the "drawing" of the laminin image, with individually numbered muscle cells, generated by the "Analyze Particles" macro within ImageJ.

Results

At the conclusion of this Honors Thesis, the study presented here remains ongoing due to requirements for additional subjects as per the National Institute of Health (NIH) grant funding this research. Because only 14 of the required 40 subjects completed the experimental protocol at the conclusion of this Honors Thesis, the data must remain blinded so as to eliminate bias in further analysis. Therefore, subject group identity (treatment vs. placebo), as well as time point of data acquisition of both MRI scans and muscle biopsies remains encoded and unknown. Consequentially, subject data cannot be allocated to groups for statistical analysis. Therefore, the discussion of results in this study is highly limited because changes in both muscle volume and muscle cross-sectional area cannot be compared with baseline values. Nevertheless, analysis was conducted on the blinded data of the 14 subjects that completed the experimental protocol, and this raw data is located in the Appendix. Figures for quadriceps cross-sectional area (CSA) and quadriceps muscle volume were reported together for each subject. Figure 4, shown below, is an example of one subject's dataset.

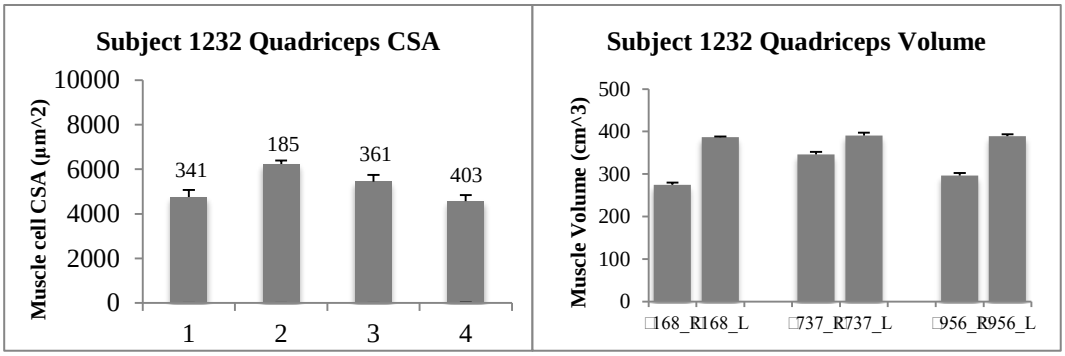


Figure 5: Quadriceps muscle cross-sectional area (CSA) and muscle volume for a single subject.

The bar graphs above display quadriceps CSA and muscle volume for Subject 1232. Each of the four muscle biopsies (pre- and post-surgery, left and right leg) used to quantify quadriceps CSA were assigned a random numerical code (1-4) by the Principle Investigator to ensure blindedness during analysis. The numbers positioned above each column in the CSA figure indicate the number of muscle cells analyzed for that biopsy. The 3-digit numbers listed below each column in the quadriceps volume figure represent a time point of MRI acquisition (2w pre-op, 2w post-op, 6w post-op), and were also encoded by the Principle Investigator. All are ordered least-to-greatest in numerical order. "R" and "L" refer to right and left leg. Error bars in both charts represent ± standard error of the mean (SEM). Data for all 14 subjects analyzed for this study are listed in the Appendix.

Discussion

The primary purpose of this study was to determine how twice daily ingestion of 20 grams of essential amino acids (EAAs) for 1 week before and 6 weeks after TKA influenced changes in muscle cell cross-sectional area following surgery. Secondarily, this study sought to confirm findings by Dreyer *et al.* that EAA supplementation attenuates muscle loss and accelerates the return of functional mobility at 6 weeks after TKA[9]. It was hypothesized that twice daily ingestion of 20 grams EAAs would attenuate muscle cell and whole-muscle atrophy of the quadriceps in both the operative and non-operative leg after TKA.

Unfortunately, because this double-blind study is incomplete and ongoing due to requirements for additional subjects stipulated in the supporting NIH grant, the results cannot be unblinded here for analysis. The concealment of subject identifying information, group allocation, and time point of data acquisition, is essential in this study in order to remove potential bias on the part of the researchers. Removing these elements before the experiment is complete would compromise its status as a randomized, placebo-controlled, double blind study and ultimately invalidate it. Subsequently, evidence-based conclusions about the effectiveness of EAA supplementation in attenuating muscle atrophy after TKA cannot be made here because they would be merely conjecture.

However, conclusions can be made about the efficacy of the analysis methods developed for this Honors Thesis. The digital strategies designed to determine muscle cell CSA and volume are accurate, objective, and yield highly reproducible results.

Both methods systematically isolate different tissue types in both contexts, allowing for easy quantification of various muscle parameters.

Intra- and inter-class variance analysis was conducted on these methods in order to determine their consistency in measurement. These metrics indicate the objectivity and reproducibility of the analysis techniques. Intra-class variance analysis was conducted on the muscle volume quantification protocol in order to determine the percent difference between measurements of the same dataset over the course of the study by a single researcher. This analysis yielded an approximately 2-3% change between quadriceps volume measurements conducted at different time points in the study. This value is low and well within the accepted range of precision for this analysis protocol (<10%).

Similarly, Inter-class variance was conducted to determine the percent difference in measurement between *separate* researchers analyzing the same dataset. These values varied between 0.5%-3.4% and were also well within the accepted range, indicating proficiency and precision of technique (<10%). Intra-class variance analysis was also conducted on the muscle fiber CSA protocol. The analysis yielded a 0.15% change between average fiber CSA for a subject's single biopsy, measured at different time points in the study. This value, too, is low and well within the accepted range of precision (<10%).

It should also be noted that over 98% of the fibers originally analyzed were analyzed again during the follow-up analysis for intra-class variance. Inter-class variance analysis could not be conducted on the muscle fiber CSA protocol because only one researcher conducted this protocol. Taken together, the low percent changes

yielded from these metrics indicate that the analysis protocols developed in this Honors Thesis are unbiased and appropriately rigorous in quantifying muscle CSA and volume.

In addition to being meticulous, both procedures are more time efficient and allow for greater analysis capabilities in comparison to past techniques. The protocol for determining CSA of muscle cells is particularly expeditious and facilitates analysis of large quantities of cells in a reasonable time frame. Of the 14 subjects included in this study, 315 cells per biopsy on average were counted and analyzed for each subject using the described method. This is a notable increase from other studies measuring muscle fiber CSA, such as Dreyer *et al.'s* 2005 study comparing satellite cell numbers in young and older men after eccentric exercise, where, on average, only 119 fibers per biopsy per subject were analyzed[47]. The expanded analysis capacity of this method empowers this study even though it is impossible to conduct meaningful statistical analysis on the blinded data here.

The protocol for determining quadriceps muscle volume is similarly efficient and dynamic. While exclusively employed to calculate quadriceps muscle volume in this study, it can easily be exploited to quantify hamstrings muscle volume (along with hamstrings intramuscular fat) and subcutaneous fat volume from the same MRI scans depicted in Figures 2 and 3. The effectiveness of this protocol is further strengthened by the wide array of capabilities of MRI technology. The "fat" and "water" images provided by the Dixon technique facilitate accurate tissue differentiation and greatly simplify the process of quantifying otherwise hard to visualize or ambiguous muscle parameters (such as intramuscular fat).

The ability to cross-reference the same image under various filters imparts confidence during analysis and allows confirmation that manual selections (such as the tracing of intermuscular septums, Figure 3, Images 3 and 4) are as detailed as possible. The combination of versatile analysis techniques with comprehensive MR image data contributes to results that are detailed and reliably reproducible.

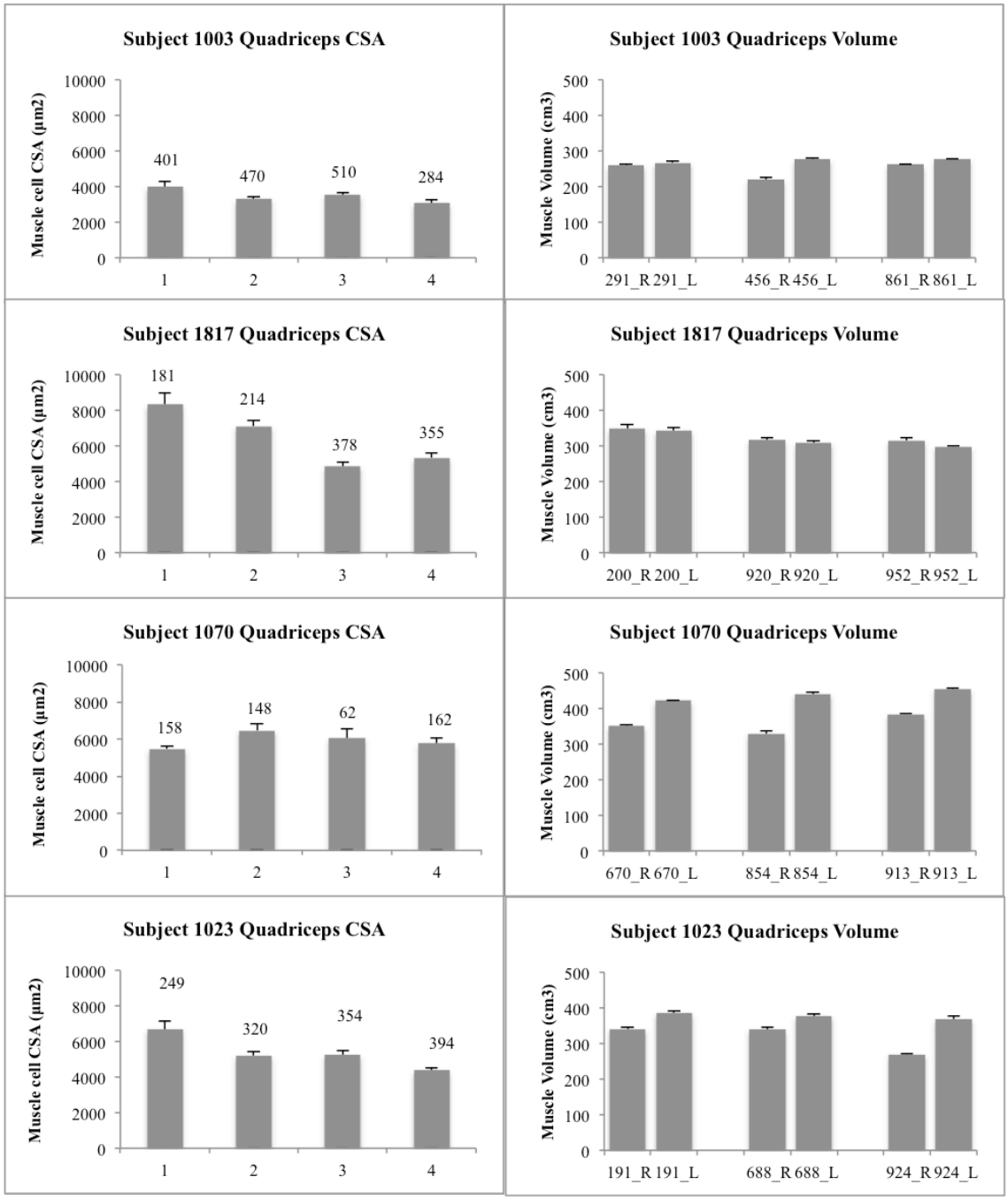
Although the successes of essential amino acid supplementation in attenuating muscle atrophy after TKA cannot be directly assessed in this study, discussion of the potential advantageousness of this strategy in a clinical context is worthwhile. Findings from the Dreyer Lab provide proof-of principle for essential amino acid supplementation in mitigating muscle atrophy and the correlated functional declines after TKA[9]. Results concerning muscle fiber cross-sectional area from this study could further support these conclusions and ostensibly provide a mechanism for the observed retention of strength and functional mobility in patients supplementing EAAs. Although complex, the positive relationship between muscle fiber cross sectional area and strength in healthy young and older individuals has been established[48,49,50,51].

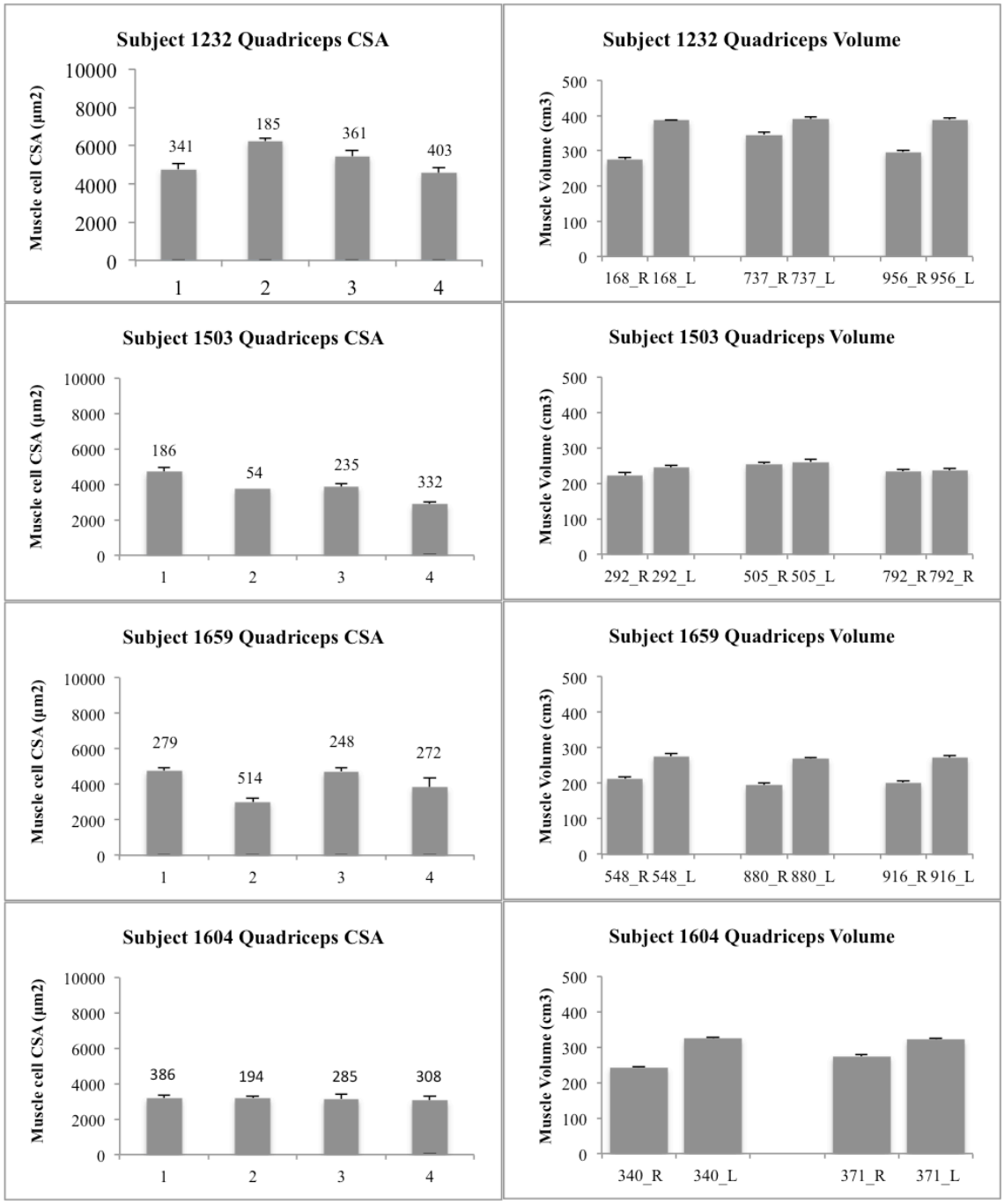
If EAA supplementation reduces muscle wasting at the cellular level, this could be a pertinent strategy in helping TKA patients retain strength, and ultimately maintain long-term functional mobility after surgery. The noninvasiveness of EAA supplementation makes it appealing in a clinical context, especially because it is not associated with adverse side effects or kidney damage.

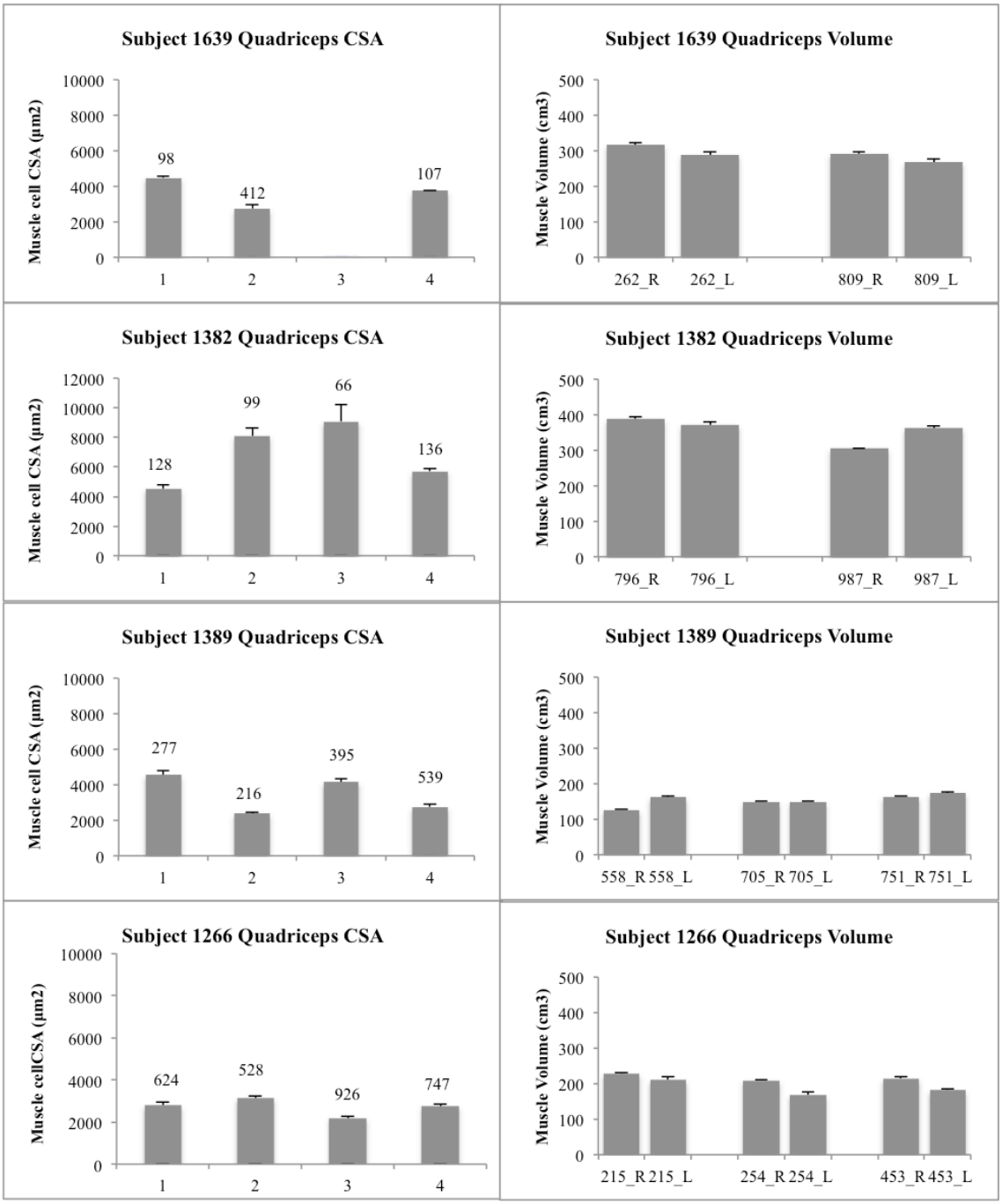
Further research is necessary to determine how EAA supplementation affects the daily lives of patients, and if it is a universally realistic approach for all TKA patients. However, it remains that EAA supplementation has the capacity to be an exceedingly simple strategy in helping the rising number of individuals undergoing total knee arthroplasty to regain functional mobility and independence after surgery.

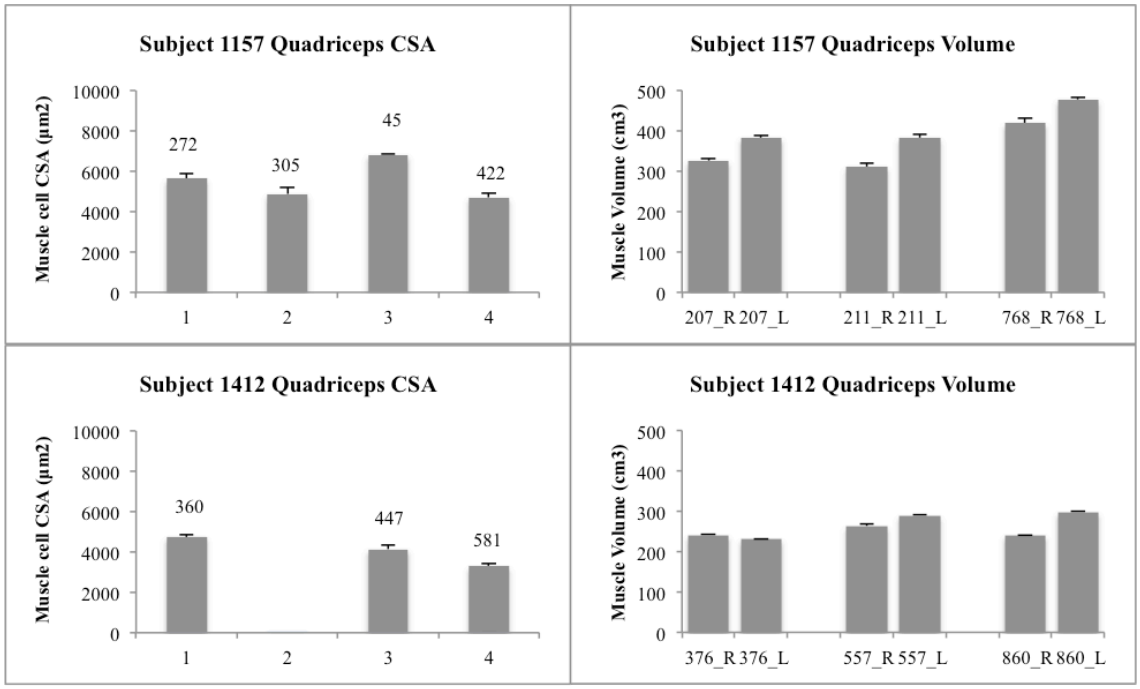
Appendix

Listed below are subject-matched graphs for both quadriceps muscle cross-sectional area (CSA) and volume. Data within these graphs remains blinded due to the stipulations described in the Results and Discussion.




Subject 1232 Quadriceps CSA
Muscle cell CSA (µm2)
10000
8000
6000
4000
2000
0
341
185
361
403
1
2
3
4
Subject 1232 Quadriceps Volume
Muscle Volume (cm3)
500
400
300
200
100
0
168_R 168_L
737_R 737_L
956_R 956_L
Subject 1503 Quadriceps CSA
Muscle cell CSA (µm2)
186
54
235
332
Subject 1503 Quadriceps Volume
Muscle Volume (cm3)
292_R 292_L
505_R 505_L
792_R 792_R
Subject 1659 Quadriceps CSA
Muscle cell CSA (µm2)
279
514
248
272
Subject 1659 Quadriceps Volume
Muscle Volume (cm3)
548_R 548_L
880_R 880_L
916_R 916_L
Subject 1604 Quadriceps CSA
Muscle cell CSA (µm2)
386
194
285
308
Subject 1604 Quadriceps Volume
Muscle Volume (cm3)
340_R
340_L
371_R
371_L


Subject 1639 Quadriceps CSA
Muscle cell CSA (µm2)
10000
8000
6000
4000
2000
0
98
412
107
1
2
3
4
Subject 1639 Quadriceps Volume
Muscle Volume (cm3)
500
400
300
200
100
0
262_R
262_L
809_R
809_L
Subject 1382 Quadriceps CSA
Muscle cell CSA (µm2)
12000
10000
8000
6000
4000
2000
0
128
99
66
136
1
2
3
4
Subject 1382 Quadriceps Volume
Muscle Volume (cm3)
500
400
300
200
100
0
796_R
796_L
987_R
987_L
Subject 1389 Quadriceps CSA
Muscle cell CSA (µm2)
10000
8000
6000
4000
2000
0
277
216
395
539
1
2
3
4
Subject 1389 Quadriceps Volume
Muscle Volume (cm3)
500
400
300
200
100
0
558_R 558_L
705_R 705_L
751_R 751_L
Subject 1266 Quadriceps CSA
Muscle cellCSA (µm2)
10000
8000
6000
4000
2000
0
624
528
926
747
1
2
3
4
Subject 1266 Quadriceps Volume
Muscle Volume (cm3)
500
400
300
200
100
0
215_R 215_L
254_R 254_L
453_R 453_L


Subject 1157 Quadriceps CSA
Muscle cell CSA (µm2)
10000
8000
6000
4000
2000
0
272
305
45
422
1
2
3
4
Subject 1157 Quadriceps Volume
Muscle Volume (cm3)
500
400
300
200
100
0
207_R 207_L
211_R 211_L
768_R 768_L
Subject 1412 Quadriceps CSA
Muscle cell CSA (µm2)
10000
8000
6000
4000
2000
0
360
447
581
1
2
3
4
Subject 1412 Quadriceps Volume
Muscle Volume (cm3)
500
400
300
200
100
0
376_R 376_L
557_R 557_L
860_R 860_L

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