ARGININE AND PROLINE METABOLISM IS ASSOCIATED WITH OLDER ADULT SKELETAL MUSCLE MASS AND ALTERED BY INFLAMMATION IN PRIMARY HUMAN MUSCLE CELLS

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ABSTRACT

Age-related loss of skeletal muscle mass (sarcopenia) adversely affects muscle tissue-specific and whole-body homeostasis, suggesting an undefined relationship between muscle mass and whole-body circulation that underlies the etiology of sarcopenia.

To investigate, 344 metabolites and analytes were measured in serum from 19 older adults. *Arginine and proline metabolism* was the pathway most closely associated with skeletal muscle index (SMI), a measure of skeletal muscle mass. Subsequently, gene expression of related transport and regulatory enzymes was measured.

Younger and older adult muscle tissue had similar inflammatory signaling and gene expression of *CAT-1* and *CAT-2*, the cationic amino acid transporters that allow arginine into muscle. However, the proinflammatory cytokine TNF-α increased *CAT-2* gene expression in differentiated human primary skeletal muscle cells. TNF-α also altered the expression of arginine metabolic genes regulating polyamine synthesis and proline production.

Changes in arginine availability and metabolism may underlie the relationship between inflammation and skeletal muscle mass.

BIOGRAPHICAL SKETCH

Diwakar Gupta grew up in Flushing, New York. He graduated from Cornell University in 2012 with a B.A. in Biology. He became interested in skeletal muscle metabolism while working as a research assistant in Dr. Anna Thalacker-Mercer's lab. In his spare time, he enjoys playing with cats and dogs and tinkering with computers. He is thankful for the support of his family, friends, and especially his labmates during graduate school.

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LIST OF ABBREVIATIONS

AGMAT, agmatinase;

ALDH4A1, pyrroline-5-carboxylate dehydrogenase;

ALDH18A1, pyrroline-5-carboxylate synthase;

ApLM, appendicular lean mass;

ARG2, arginase 2;

ASL, argininosuccinate lyase;

ASS1, argininosuccinate synthase;

B2M, beta-2-microglobulin;

CAT-1 and *CAT-2*, cationic amino acid transporter 1 and 2;

cDNA, complementary DNA;

CKM, creatine kinase, muscle;

DM, differentiation media;

DXA, dual x-ray absorptiometry;

FACS, fluorescence activated cell sorting;

FBS, fetal bovine serum;

FDR, false discovery rate;

GLS, glutaminase;

GM, growth medium;

GP130, interleukin 6 signal transducer;

HMB, β-hydroxy β-methylbutyrate;

HMRU, Human Metabolic Research Unit at Cornell University;

HPLC-MS, high performance liquid chromatography mass spectrometry;

LBM, lean body mass;

MPB, muscle protein breakdown;

MPS, muscle protein synthesis;

mTOR, mechanistic target of rapamycin;

MuIS, muscle inflammatory susceptibility;

NFKB1, nuclear factor kappa B subunit 1;

NOS2, nitric oxide synthase 2 (inducible);

NMR, nuclear magnetic resonance;

OAT, ornithine aminotransferase;

ODC1, ornithine decarboxylase 1;

OTC, ornithine carbamoyltransferase;

PBS, phosphate-buffered saline;

PPIA, peptidylprolyl isomerase A;

PYCR1, pyrroline-5-carboxylate reductase 1;

P4HA1, prolyl 4-hydroxylase subunit alpha 1;

qPCR, quantitative polymerase chain reaction;

RPLP0, ribosomal protein lateral stalk subunit P0;

RT, resistance exercise training;

SMI, skeletal muscle index;

SMOX, spermine oxidase;

SRM, spermidine synthase;

STAT3, signal transducer and activator of transcription;

TBP, TATA-box binding protein;

TNF-α, tumor necrosis factor alpha

TNF-R1, TNF receptor 1;

UAB, University of Alabama at Birmingham;

WPC80, 80% whey protein concentrate;

4E-BP1, eukaryotic translation initiation factor 4E binding protein 1.

CHAPTER 1
BACKGROUND

Sarcopenia

In the U.S., adults over the age of 65 are a growing demographic: they comprised 13.7% of the population in 2012, but this percentage will rise to 21% by 2040 [1]. Life-expectancy at birth has increased by four years in the last two decades [2], but later stages of life are characterized by decreased quality of life [3]. This decrease in quality of life is linked to sarcopenia, the involuntary loss of skeletal muscle mass and associated decline in strength and function. Sarcopenia begins as early as the third decade of life [4] and increases the risk of frailty, injury, and disability in older adults [5, 6]. Low skeletal muscle mass in older adults is associated with difficulty performing activities of daily living (e.g., eating, bathing, climbing stairs, cooking, or shopping [7, 8]), suggesting that skeletal muscle mass and function are important for living independently and maintaining a high quality of life. Therapies to help maintain skeletal muscle mass with aging are essential; however, they are poorly defined. Development of efficacious therapies for ameliorating skeletal muscle deterioration with aging requires understanding the intrinsic and extrinsic factors that impact skeletal muscle health.

Understanding sarcopenia is challenging because aging is a multi-factorial process and aging skeletal muscle differs from young muscle in a number of ways. Compared to younger adults, older adult skeletal muscle has impaired protein balance that favors atrophy. This atrophy can be attributed, in part, to blunted anabolic responses to hypertrophic stimuli [9] and potentially impaired regulation of protein

breakdown as suggested by increased gene expression of ubiquitin ligases that regulate protein breakdown [10]. Older adult skeletal muscle also exhibits loss of muscle fibers (i.e. multinucleated skeletal muscle cells) and increased atrophy of the type II glycolytic muscle fibers that provide rapid contractile activity [11]. The disruption of skeletal muscle homeostasis that occurs with aging has been attributed to factors both intrinsic and extrinsic to the skeletal muscle; however, the etiology and identification of therapeutic targets to attenuate sarcopenia remains ambiguous.

Skeletal muscle—a metabolic organ system

As the largest metabolic organ system in the human body, skeletal muscle metabolism affects not only skeletal muscle health, but also the health of the entire body. Skeletal muscle is the largest glucose sink; alterations in glucose uptake and metabolism by the muscle can ultimately affect glucose homeostasis throughout the body. Additionally, muscle releases myokines, such as interleukin 6 (IL-6), which act in an autocrine, paracrine, and endocrine manner [12]. Similarly, skeletal muscle is affected by circulating factors, such as adipokines released by adipose tissue [13]. Further, a parabiosis study in mice showed that serum from aged mice causes young mouse muscle satellite cells (i.e., muscle stem cells) to develop into fibrous connective tissue instead of contractile myocytes [14]. This study demonstrates that there are circulating factors which contribute to skeletal muscle aging, but their identity remains unknown. By measuring circulating factors (e.g., metabolites and analytes), it is

possible to study this complex network of interactions and generate testable hypotheses regarding the interactions between skeletal muscle and other systems.

Metabolomics

Metabolism involves complex networks of interconnected biochemical reactions which produce the vast assortment of metabolites needed for various biological functions. A change in the concentration of one metabolite can launch a cascade of downstream effects. Traditional metabolic studies focus on a small number of metabolites individually selected for measurement. Metabolomics involves the measurement and analysis of large quantities of metabolites to understand metabolism. Technologies such as nuclear magnetic resonance (NMR) and high resolution mass spectrometry allow researchers to measure hundreds of metabolites simultaneously. Traditional metabolic studies rely on selecting the correct metabolites to measure in order to determine some of the changes caused by a metabolic perturbation. However, metabolomics makes it possible to determine how a perturbation affects a metabolic network(s) as a whole and thus, is a useful tool for the identification of biomarkers and early disease states [15-17]. Metabolomics is also used for hypothesis generation: identifying metabolites that are associated with a particular condition or disease state, so that they can be studied in-depth individually [18]. Overall, metabolomics has the potential to allow the identification of metabolic pathways that are linked to sarcopenia.

To identify whole body metabolic disturbances that are associated with muscle mass and likely underlie sarcopenia, we will use serum metabolomics to identify circulating metabolites that are associated with SMI in older human adults.

Skeletal muscle inflammation—the good and the bad

Inflammation is a key factor in skeletal muscle regeneration and deterioration. Exercise is associated with an acute inflammatory response, characterized by increased production and secretion of cytokines such as IL-6 [19]. This acute inflammatory response is thought to be critical for muscle regeneration and the beneficial effects of exercise [20]. In contrast, chronic low-grade inflammation is positively associated with aging [21] and metabolic disease [22] and negatively associated with physical activity [23]. Chronic inflammation leads to pathological remodeling of the skeletal muscle (e.g. loss of myofibers and gain of fibrotic and adipose tissues). The proinflammatory cytokine TNF-α is a signaling molecule and an initiator of inflammation [24]. In older adults, circulating levels of TNF-α are positively associated with diminished muscle strength [25, 26] and mortality [27, 28]. TNF-α activates the transcription factor NF-κB, which regulates the expression of genes that govern skeletal muscle growth and differentiation [29]. The activation of NF-κB is associated with multiple skeletal muscle disorders, including Duchenne muscular dystrophy, inflammatory myopathies, cancer cachexia, and disuse atrophy [30]. Although there is a known association between inflammatory signaling and

skeletal muscle health, the mechanisms governing this relationship are not fully understood.

Arginine

Arginine is a conditionally-essential amino acid that the body can synthesize from other metabolites. Arginine is primarily synthesized from citrulline in the kidney by the enzyme argininosuccinate synthase [31]. Citrulline is formed from glutamine, glutamate, and proline in the gut [31]. When these necessary precursors for arginine synthesis are absent, arginine needs to be supplied exogenously.

Arginine supplementation may be an effective therapy to help maintain skeletal muscle mass. Research has demonstrated that supplements containing arginine have beneficial, hypertrophic effects on skeletal muscle [32-34]. Older women who consumed an 8.5 g mixture of arginine, β-hydroxy β-methylbutyrate (HMB), and lysine for 12 weeks had 20% higher whole-body protein synthesis compared to patients who consumed an isocaloric maltodextrin placebo, although there was no significant difference between groups in net protein gain [32]. The older women also had increased leg strength and functionality [32]. Adults with chronic inflammatory diseases also benefit from supplements containing arginine. Adults with HIV who consumed a supplement consisting of arginine, HMB, and glutamine daily for 8 weeks increased their lean body mass (LBM) by 2.55 kg, while subjects who received a maltodextrin placebo lost 0.70 kg LBM [33]. In addition, adults with cancer cachexia who consumed a similar supplement (as the adults in the HIV study) for 24 weeks

increased their fat-free mass 1.12 kg by week 4 and 1.60 kg by week 24 [34]. Intriguingly, healthy, young athletes do not have improved performance with supplemental arginine [35-38]. There is conflicting evidence to suggest arginine may delay fatigue during aerobic exercise in young male adults, but arginine appears to be acting on endothelial cells to improve blood flow during exercise, not on skeletal muscle cells [39]. Supplemental arginine is beneficial for muscle health in populations expected to have elevated inflammation, suggesting that inflammation may increase demand for arginine or decrease arginine synthesis.

Arginine and inflammation

The mechanism underlying the relationship between inflammation and arginine may involve the proinflammatory cytokine TNF-α. TNF-α is known to regulate arginine metabolism in rat neuronal cells, rat cardiac myocytes, and human vascular smooth muscle [40] by increasing gene expression of the cationic amino acid transporter (*CAT-2*). The CAT isoforms (CAT-1, CAT-2A, and CAT-2B) allow arginine, lysine, and ornithine to pass through the plasma membrane. TNF-α increases the gene expression of *CAT-2* in human endothelial cells by activating the NF-κB signaling pathway [40], and NF-κB DNA-binding activity is increased by low arginine availability [41]. It is unknown if TNF-α also stimulates the gene expression of *CAT-2* in human skeletal muscle cells. We predict that TNF-α increases *CAT-2* gene expression in human skeletal muscle cells, increasing arginine uptake. We also predict that increased arginine availability alters arginine metabolism, changing the relative

distribution of arginine metabolites and changing skeletal muscle metabolic and physiologic processes.

Arginine and protein metabolism

The hypertrophic effects of supplements containing arginine in adults with chronic inflammatory diseases suggest that one of the metabolic pathways affected by arginine availability is the regulation of protein balance. Supplements containing arginine may improve skeletal muscle mass by impacting protein balance, either by stimulating muscle protein synthesis (MPS), inhibiting muscle protein breakdown (MPB), or both. MPS is regulated by the mammalian target of rapamycin, which forms a complex (mTORC1) that integrates signals denoting nutritional status, energy status, and the presence of growth factors [42]. For example, amino acids and exercise are positive regulators of mTORC1 [43-46]. Although some of the factors that regulate MPS have been identified, the atrophic mechanisms underlying sarcopenia have not been fully elucidated. One contributor to sarcopenia may be a diminished hypertrophic response to anabolic stimuli such as essential amino acids [9]. Essential amino acids stimulate protein synthesis in younger adults, but older adults have a blunted response to this stimulus [9]. Heightened inflammation, induced by infusion of TNF-α, decreases MPS [47]. Additionally, animal and cell culture studies provide evidence that arginine alone can increase the phosphorylation of mTORC1, increase MPS, and increase myotube diameter [48, 49]. The interaction between arginine and mTORC1 in

these studies may be mediated by the lysosomal protein SLC38A9, which is necessary for the activation of mTORC1 by arginine [50].

Skeletal muscle mass is also shaped by protein breakdown. Muscle protein breakdown (MPB) is difficult to quantify directly; therefore, regulators of protein breakdown such as E3 ubiquitin ligases are measured as a surrogate for MPB. Compared to younger adults, older adults have elevated muscle inflammatory signaling [51] that may disrupt MPB and thus protein balance. Compared to young women, older females have higher gene expression of the FoxO-regulated E3 ubiquitin ligases *MURF-1* and *atrogin-1* at rest and after resistance exercise, respectively [10]. TNF-α increases protein expression of ubiquitin, MuRF-1, and atrogin-1 in rat L6 myotubes [52], and in murine C2C12 myotubes, exposure to TNF-α increases breakdown of adult myosin heavy chain, indicative of increased protein catabolism [53]. Arginine ameliorates increased gene expression of *MURF-1* and *atrogin-1* in rat muscle induced by hindlimb suspension [54]. Low arginine availability increases the DNA-binding and activation of NF-κB [41]. Since NF-κB increases the gene expression of *MURF-1* and *atrogin-1* in addition to *CAT-2* [55], increased arginine flux through CAT-2 may be part of a negative feedback loop that limits inflammatory signaling.

Overall, these studies suggest potential pathways through which arginine may have protective effects against muscle protein imbalance. Arginine may activate mTORC1 to increase protein synthesis, ameliorate protein breakdown induced by NF-κB or FoxO, or it may be converted into another metabolite that affects skeletal

muscle metabolism directly. However, it is unclear which pathways are active during chronic inflammation.

Arginine metabolism

Arginine may also indirectly impact skeletal muscle health by serving as a precursor to several different metabolites which can affect skeletal muscle metabolism. Arginine is required for the production of nitric oxide, a signaling molecule that regulates mitochondrial biogenesis [56]. Nitric oxide can bind to proteins in myofibrils to protect them from degradation by calpains, but aging is associated with reduced protein expression of neuronal nitric oxide synthase in mouse skeletal muscle [57]. Arginine can also be converted into polyamines, metabolites that regulate cell proliferation [58]. The enzyme ornithine decarboxylase 1 catalyzes the synthesis of the polyamine putrescine and regulates myoblast differentiation [59]. The polyamine spermine is a ligand for spermine oxidase, which regulates genes controlling muscle atrophy [60]. Arginine can also be converted to proline and hydroxyproline, amino acids which are abundant in collagen, a key component of wound healing [61]. It is not known if changes in the production of these metabolites are linked to inflammation or sarcopenia.

The relationship between inflammation and arginine in skeletal muscle suggests that older adults with heightened inflammation may have dysfunctional arginine metabolism. ***Understanding how these alterations in arginine metabolism***

affect skeletal muscle metabolic and physiologic processes is necessary to inform the development of therapies to improve skeletal muscle health in older adults.

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CHAPTER 2

Arginine and proline metabolism is associated with older adult skeletal muscle mass and altered by inflammation in primary human muscle cells

Abstract

Background: Age-related loss of skeletal muscle mass (i.e. sarcopenia) adversely affects muscle tissue-specific and whole-body homeostasis, suggesting an undefined relationship between muscle mass and whole-body circulation. Characterizing this relationship will further our understanding of the etiology of sarcopenia.

Objective: Identify circulating metabolites and metabolic pathways correlated with skeletal muscle index (SMI), a normalized measure of skeletal muscle mass.

Design: Serum and appendicular lean tissue measurements were collected before and after a 35-week resistance exercise training (RT) intervention in 19 older adults. Using high resolution mass spectrometry and immunoassays, 344 unique metabolites and analytes were analyzed from serum. MetaboAnalyst was used to identify metabolic pathways associated with SMI. Follow-up qPCR analysis of the most significant metabolic pathway was conducted on genes for transport and metabolic enzymes using skeletal muscle tissue and differentiated primary human skeletal muscle cell (myotube) cultures.

Results: Arginine and Proline Metabolism was most strongly associated with SMI before and after RT. In the follow-up analysis, expression of arginine and proline transport and metabolism genes, cationic amino acid transporter 2, ornithine decarboxylase 1, pyrroline-5-carboxylate reductase 1, spermidine synthase, creatine

kinase, prolyl 4-hydroxylase subunit alpha 1, and ornithine aminotransferase were altered by inflammation in human myotubes.

Conclusions: Lower levels of arginine and proline metabolites, observed among older adults in vivo, are consistent with reduced expression of related metabolic enzymes consequent to induced inflammation in vitro. These results suggest that therapies to manipulate arginine availability could improve skeletal muscle health in populations with elevated muscle inflammation, including many older adults.

Introduction

The involuntary age-related loss of skeletal muscle mass (sarcopenia), as well as concurrent fat and fibrotic tissue accumulation within and around skeletal muscle, increase the risk for functional and metabolic deterioration [1]. Age-related changes in skeletal muscle morphology and physiology [2-4] accelerate with advancing age, and are accentuated by intrinsic [5-8] and extrinsic factors [9]. Factors influencing muscular changes have been identified in the single myofiber, the skeletal muscle organ system, and the whole organism. However, aging skeletal muscle is complex and multifactorial, and the molecular mechanisms instituting adverse changes with age are unclear.

Skeletal muscle remodeling and deterioration with aging adversely affect muscle tissue-specific and whole-body homeostasis [10]. Conversely, whole-body homeostasis can adversely affect muscle mass [11], which suggests an undefined relationship between skeletal muscle mass and whole-body circulation. Studies using parabiosis [12] and primary progenitor cells [12, 13] have demonstrated that factors intrinsic to serum from older adults lead to impaired skeletal muscle regeneration; however, what circulating factors contribute to these observed age-related differences are ambiguous. These combined findings strongly suggest a circulating metabolic signature, indicative of reduced skeletal muscle mass and poor muscle health, but specific metabolites and analytes are largely unknown and need to be identified and characterized.

Metabolomics, the comprehensive, quantitative measurement of endogenous metabolites from biological specimens, is a potential tool to classify samples, generate

hypotheses, and identify potential mechanisms and biomarkers [14-16]. Metabolomics has been employed to understand metabolic processes and identify biomarkers of chronic disease (e.g. diabetes, cancer, etc.), physiological states (e.g. cancer cachexia, aerobic capacity, subcutaneous and ectopic skeletal muscle fat, etc.), therapy responses (e.g. caloric restriction), and exercise training adaptations (e.g. strength-endurance training). The objective of this study was to examine serum metabolites, using metabolomics, in older adults before and after resistance exercise training (RT) and identify metabolic pathways that are most closely related to skeletal muscle index (SMI), a widely used indicator of sarcopenia [17]. Follow-up analyses were aimed at interrogating identified metabolic pathways associated with SMI to further our understanding of potential mechanisms driving muscle atrophy with aging.

Materials and Methods

Subjects

The metabolomics study was conducted as a secondary analysis of a subset of collected serum samples, dietary records, and body composition data (see **Figure 1** for study design) on 19 older men (n = 6) and women (n = 13) aged 60 - 75 y (**Table 1**). For the parent resistance exercise training (RT) study, all subjects were recruited from the greater Birmingham, Alabama area. A health history and physical activity questionnaire as well as a comprehensive physical exam were completed by all subjects during screening. Subjects also passed a diagnostic, graded exercise stress test with 12-lead electrocardiogram reviewed by a cardiologist. Subjects were excluded from the study if they reported any musculoskeletal or other disorders that would have affected their ability to complete exercise training and testing for the study; were obese (BMI > 30.0 kg/m^2); performed knee extensor RT within the past 5 y; or were treated with exogenous testosterone or other pharmacological interventions known to influence muscle mass or muscle recovery.

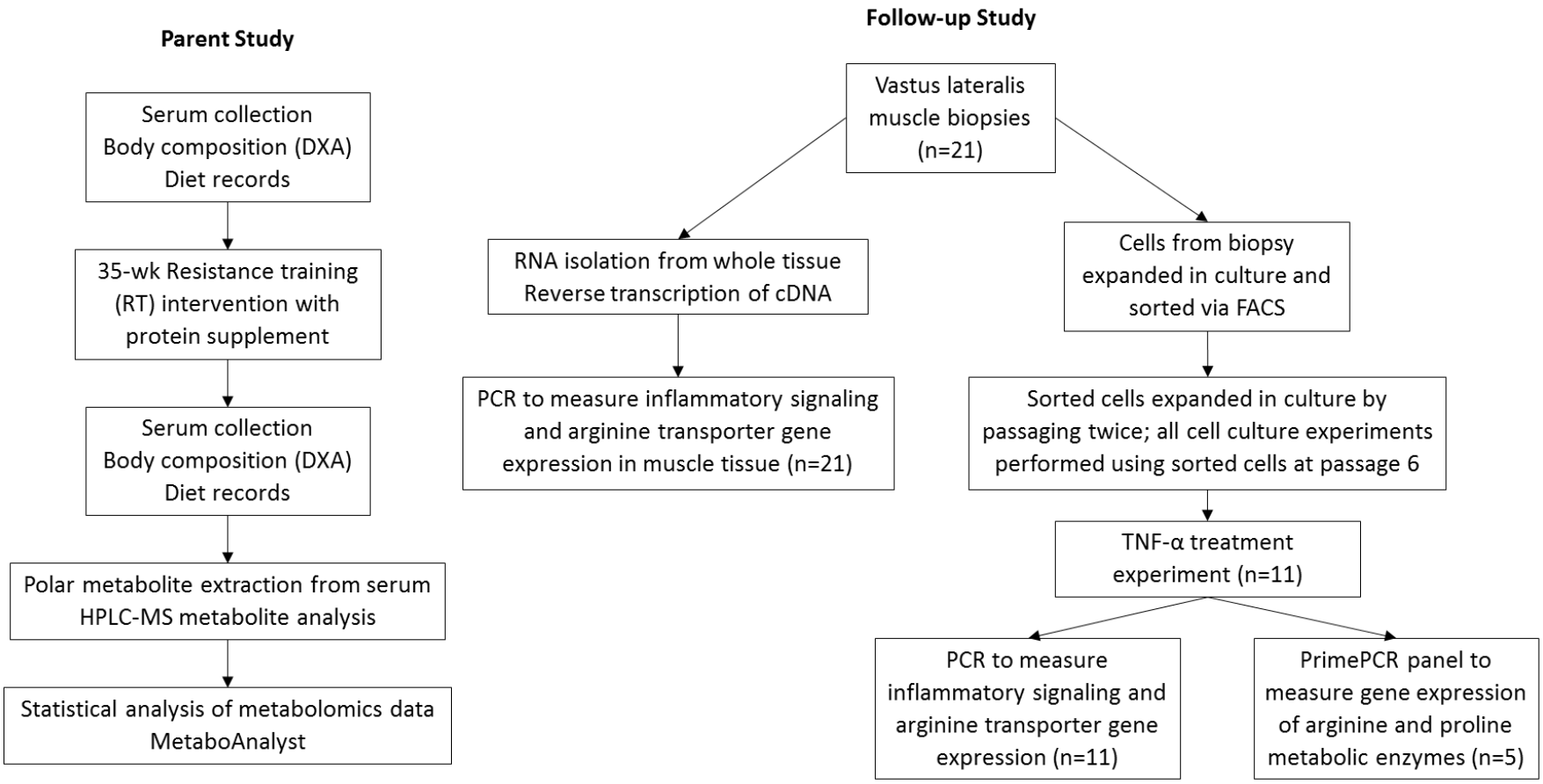

Figure 1: Experimental design of parent metabolomics study (conducted at UAB) and follow-up study (conducted at Cornell). DXA, dual x-ray absorptiometry; FACS, fluorescence activated cell sorting; HPLC-MS, high-performance liquid chromatography mass spectrometry; RT, resistance exercise training; UAB, University of Alabama at Birmingham

Table 1. Subject characteristics for resistance exercise training (RT) intervention

	Baseline	RT
Subjects (females)	19 (13)	19 (13)
# Sarcopenic	2	1
Age, *y*	65 ± 1	66 ± 1***
Weight, *kg*	77.7 ± 2.9	78.1 ± 2.9
BMI	26.1 ± 0.7	26.3 ± 0.7
ApLM, *kg*	21.1 ± 1.1	21.6 ± 1.1**
SMI, kg/m^2	7.0 ± 0.3	7.2 ± 0.3**
Lean body mass, *kg*	46.3 ± 2.0	47.4 ± 1.9***
Fat mass, *kg*	28.0 ± 1.7	27.5 ± 1.6

Means ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, significantly different from baseline (paired t-test). ApLM, appendicular lean mass; RT, resistance exercise training; SMI, skeletal muscle index (ApLM divided by height squared).

Follow-up experiments to the metabolomics analysis (see Figure 1 for study design) involved collection of both skeletal muscle tissue and/or human primary satellite cells from 11 younger men (n = 6) and women (n = 5) aged 21 - 39 y and 10 older men (n = 4) and women (n = 6) aged 68 - 80 y who were recruited from the Tompkins County, New York area. Individuals in the follow-up study completed a comprehensive health history and physical activity questionnaire that was reviewed by a nurse practitioner. Individuals were excluded for any chronic end-stage disease expected to limit life-expectancy to less than one year, induce anorexia, or restrict physical activity; if they had a seated resting systolic blood pressure > 140 mm Hg or

diastolic blood pressure > 90 mm Hg; or if they were currently adhering to a weight-reduction diet.

Studies were approved by the Institutional Review Boards of the University of Alabama at Birmingham (UAB) and Cornell University, respectively. All subjects provided written informed consent prior to participation.

METABOLOMICS STUDY

Resistance training and dietary analysis

All subjects underwent 35 weeks of supervised, whole-body, progressive RT. Subjects were randomly assigned into one of four RT programs [high-resistance concentric-eccentric training (H) 3 d/wk (HHH); H training 2 d/wk (HH); 3 d/wk mixed model training consisting of H training 2 d/wk separated by one bout of low-resistance, high-velocity, concentric only (L) training (HLH); or 2 d/wk mixed model consisting of H training 1 d/wk and L training 1 d/wk (HL)]. All subjects were asked to maintain their habitual diet throughout the RT study. Four-day diet records were collected at the beginning and end of the 35-wk RT to monitor habitual intake. Diet records were analyzed using Nutrition Data System for Research 2013 (University of Minnesota), a dietary analysis program. Diet record data were used to determine whether dietary macronutrient or micronutrient intake affected SMI or circulating metabolite concentrations. In addition to their habitual diet, subjects consumed 0.3 g per kg body weight of 80% whey protein concentrate (WPC80, Agri-Mark, Methuen, Massachusetts) immediately before and after each exercise session throughout the 35-wk RT. All subjects consumed a total of 0.6 g of WPC80 per kg body weight every

Monday, Wednesday, and Friday throughout the 35-wk study, including participants assigned to RT 2 d/wk.

Anthropometrics and body composition

Prior to and after the 35-wk RT, a stadiometer was used to measure height, and a dual x-ray absorptiometry (DXA) scan was used to measure fat, lean (total and appendicular), and bone masses. SMI was calculated as appendicular lean mass (kg) / height^2 (m^2).

Serum metabolomics and analyte measurements

Serum collected from participants in each of the four RT training programs [HHH (n = 4), HH (n = 5), HLH (n = 5), HL (n=5)] was analyzed for metabolite concentrations. Before and after the 35-wk intervention, subjects were asked to complete an overnight fast and report to the metabolic unit at UAB. Serum was collected via venipuncture by a trained nurse. Serum was aliquotted and stored at -80°C. For polar metabolite extraction, 25 µL of serum were extracted with 500 µL of 80% methanol (HPLC grade) chilled over liquid nitrogen vapor and centrifuged [18]. Supernatants containing the polar solvents were dried and concentrated using a vacuum concentrator (SpeedVac Concentrator, Savant) [18]. Concentrated samples were reconstituted in water and acetonitrile, then analyzed using high-resolution mass spectrometry coupled to liquid chromatography (HPLC-MS) to measure 338 metabolites [19]. Immunoassay was used to measure seven analytes: glucose, TNF-α,

IL-6, IL-1β, IL-10, leptin, and insulin using the Dimension Xpand (Siemens) and the Magpix (Luminex), following manufacturer protocols.

Statistical analysis—metabolomics study

Statistical analysis was performed using the R software environment (R Core Team, Vienna, Austria), except where noted. A paired t-test was used to identify subject characteristics, circulating metabolites, and dietary components that changed during the intervention. The association between SMI and a circulating metabolite or a dietary nutrient was calculated using Pearson's correlation, r. The false discovery rate (FDR) was controlled using the Benjamini-Hochberg procedure to adjust for multiple comparisons, and adjusted p-values < 0.05 were considered significant [20, 21].

The Pathway Analysis function of MetaboAnalyst 3.0 was used to identify biochemical pathways that were associated with SMI [22, 23]. If a metabolite had multiple isomers that could not be distinguished by mass spectrometry, the concentrations for that metabolite were inputted multiple times, labeled as each different isomer. MetaboAnalyst recognized 330 compounds, including multiple isomers, from the dataset. Interquartile range filtering removed the 5% of compounds that showed the lowest variation with SMI. No sample normalization, data transformation, or data scaling was applied. The *Homo Sapiens* pathway library was used by MetaboAnalyst for analysis. Pathway topology analysis determined the importance of each measured metabolite in a biochemical pathway by counting the number of shortest paths that passed through each metabolite (relative betweenness centrality). Then the importance of the measured metabolites was normalized by the

total importance of all the metabolites in the pathway to determine the pathway impact of each measured metabolite.

FOLLOW-UP STUDY

Muscle biopsies

Skeletal muscle biopsies were performed by a nurse practitioner in the Human Metabolic Research Unit (HMRU) at Cornell University. After an overnight fast, subjects reported to the HMRU, vitals were measured, and subjects rested in the supine position. A percutaneous needle biopsy of the vastus lateralis was taken using a 5.0 mm Bergstrom biopsy needle with suction under local anesthetic (1% lidocaine). Tissue was quickly blotted with sterile gauze, and visible adipose and connective tissues were removed. Skeletal muscle aliquots were either snap frozen in liquid nitrogen and stored at -80°C or were placed in Gibco® Hibernate®A (Thermo Fisher Scientific, Waltham, Massachusetts) for harvesting primary satellite cells.

Primary human myoblast purification and cell culture

To harvest primary satellite cells, a 70-100 mg portion of biopsy tissue was cleaned of extraneous fascia and adipose tissue and washed via gravity with Dulbecco's PBS. Tissue was dissociated via incubation at 37°C in DMEM (Gibco, Thermo Fisher, Waltham, Massachusetts) containing 10 mM HEPES, 0.03% EDTA, 0.12% Pronase E with periodic titurating until a homogeneous suspension was achieved. The cell suspension was then passed through a 70 µm cell strainer into an equal volume of FBS (GE Healthcare Hyclone, Little Chalfont, United Kingdom), and

centrifuged at room temperature for 5 minutes at 300 rcf. The resulting pellet was resuspended in myoblast growth medium [(GM) Ham's F12 (Gibco, Thermo Fisher, Waltham, Massachusetts) supplemented with 20% FBS, 1% penicillin/streptomycin (Gibco, Thermo Fisher, Waltham, Massachusetts), and 5 ng/mL recombinant human basic fibroblast growth factor (Promega, Madison, Wisconsin)]. Cells were depleted of fibroblasts by preplating followed by culture on Type I-collagen (Corning, Corning, New York) coated culture dishes at an initial cell confluency of ~15%. After 24 hours, media was replaced with fresh GM and then further replenished every 48 hours. When cultures reached 75-80% confluence, cells were detached from the plate using 0.05% Trypsin-EDTA (Gibco, Thermo Fisher, Waltham, Massachusetts). Cultures were expanded through passage three followed by cryopreservation in 10% DMSO + GM.

Although no testing for mycoplasma contamination was performed, pure populations of primary myoblasts were obtained by rapidly thawing approximately 1-1.5 million passage four cells and labeling with fluorescently conjugated antibodies to cell surface antigens CD56 (NCAM; PE-Cy7 Mouse anti Human CD56 (Clone B159), BD Pharmigen, San Jose, California) and CD29 (integrin β1; Alexa Fluor 488 anti-human CD29, Biolegend, San Diego, California) and the viability stain 7-Aminoactinomycin D (7-AAD, eBioscience, San Diego, California) [24]. Live CD56+/29+ cells were collected via fluorescence activated cell sorting (FACS) using a BD FACS Aria Fusion flow cytometer (Biotechnology Resource Center, Cornell University) and subcultured once before cryopreservation.

Freshly thawed, passage 6, CD56+/CD29+ cells from young males (n = 6) and young females (n = 5) were seeded at a density of approximately 15%, cultured in GM

until ~80% confluent, rinsed with Ham's F12 media to remove any residual serum or growth factors, and switched to differentiation media [(DM) Ham's F12 supplemented with 2% heat inactivated equine serum (Gibco, Thermo Fisher, Waltham, Massachusetts), 1% penicillin/streptomycin]. DM was replenished every 24 hours. After 72 hours, cells were then incubated for an additional 48 h in DM with or without 10 ng/mL of TNF-α (recombinant human TNF-α, Merck Millipore, Darmstadt, Germany) before cells were harvested.

RNA isolation from tissue homogenates and primary cell culture

RNA was obtained from snap frozen skeletal muscle tissue (20-30 mg) via pulverization using a liquid nitrogen-cooled Bessman tissue pulverizer followed by homogenization in TRIzol (Life Technologies, Carlsbad, California) with a rotor-stator homogenizer, following the manufacturer's protocol with the following modifications. Phase separation was achieved with 1-bromo, 3-chloropropane (120 µL per 1 mL TRIzol) followed by a 10 minute incubation at room temperature and centrifugation at 13,000 g for 15 minutes in a 4°C centrifuge. Following the addition of isopropanol and incubation at room temperature for 10 minutes, lysates were centrifuged at 12,600 g for 15 minutes in a 4°C centrifuge. The resulting RNA pellet was washed 3 times in 75% ethanol, each followed by centrifugation at 4°C and 10,000 g for 5 minutes.

For cultured cells, RNA was extracted and purified using the E.Z.N.A Total RNA Kit (Omega Biotek, Norcross, Georgia) including an on-Column DNAse digest

(Qiagen, Hilden, Germany). Total RNA quantity and quality was assessed using a Spectramax M3 spectrophotometer (Molecular Devices).

Quantitative polymerase chain reaction (qPCR)

qPCR was performed using TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, Massachusetts) for selected genes. cDNA was reverse transcribed from both skeletal muscle and cultured cell RNA using High Capacity Reverse Transcriptase (Applied Biosystems, Foster City, California). The relative gene expressions of *TNF-α* (Hs01113624_g1), TNF receptor 1 (*TNF-R1*, Hs00533560_m1), nuclear factor kappa B subunit 1 (*NFKB1*, HS00765730_m1), signal transducer and activator of transcription (*STAT3*, Hs00374280_m1), *IL-6* (Hs00985639_m1), *IL-6 receptor (Hs00794121_m1),* IL-6 signal transducer (*GP130*, Hs00174360_m1), and the cationic amino acid transporters (*CAT-1*, Hs00931450_m1, and *CAT-2*, Hs00952727_m1) were measured with TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, California) on a LightCycler 480 (Roche) running the following cycling program: 50°C, 2 min; 95°C, 5 min; 40 cycles of 95°C, 10 sec and 60°C, 30 sec. Gene expression was normalized using human ribosomal RNA *18s* (Hs99999901_s1) or TATA-box binding protein (*TBP*, Hs00427621_m1) as an endogenous control.

PrimePCR Panel

RNA from cultured cells from young females (n = 5) was used to measure the expression of genes that regulate arginine metabolism (**Table 2**). The iScript advanced

Table 2. Genes that were measured for expression using the PrimePCR Panel

Symbol	Gene
AGMAT	agmatinase
ALDH18A1	pyrroline-5-carboxylate synthase
ALDH4A1	pyrroline-5-carboxylate dehydrogenase
ARG2	arginase 2
ASL	argininosuccinate lyase
ASS1	argininosuccinate synthase
B2M	beta-2-microglobulin
CKM	creatine kinase, muscle
GLS	glutaminase
NOS2	nitric oxide synthase 2
OAT	ornithine aminotransferase
ODC1	ornithine decarboxylase 1
OTC	ornithine carbamoyltransferase
P4HA1	prolyl 4-hydroxylase subunit alpha 1
PPIA	peptidylprolyl isomerase A
PYCR1	pyrroline-5-carboxylate reductase 1
RPLP0	ribosomal protein lateral stalk subunit P0
SMOX	spermine oxidase
SRM	spermidine synthase

cDNA synthesis kit (Bio-Rad, Hercules, California) was used to synthesize cDNA from 0.32 μg of RNA. The reaction was incubated at 42°C for 30 min and then heated to 85°C for 5 min to inactivate the reverse transcriptase. To perform qPCR, cDNA was added to the 2x SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, California) along with RNase-free water to create the qPCR mixture. To load 10 ng of cDNA to each well, 20 μL of the qPCR mixture were added to each well of the custom PrimePCR Panel. The plate was sealed and centrifuged to remove bubbles. The

LightCycler 480 (Roche) ran the following cycling program: 95°C, 2 min; 40 cycles of 95°C, 5 sec and 60°C, 30 sec, with a ramp rate of 4.4°C/s. After completing the run, dissociation curve analysis was performed by increasing the temperature from 60°C to 95°C by 4.4°C/sec. The presence of a single peak in each reaction at temperatures greater than 80°C was used to verify qPCR specificity.

The NormFinder program was used to select housekeepers for normalizing gene expression measured with the PCR panel [25]. Gene expression was normalized using the geometric mean of beta-2-microglobulin (*B2M*) and ribosomal protein lateral stalk subunit P0 (*RPLP0*) as endogenous controls.

Statistical analysis—follow-up study

A two-factor ANOVA was computed in Prism 4 (GraphPad, La Jolla, California) to determine the effects of age and sex on gene expression in skeletal muscle tissue. A paired t-test was computed in Excel 2013 (Microsoft, Redmond, Washington) to determine the effect of TNF-α on *in vitro* gene expression measured with Taqman Gene Expression Assays or the PrimePCR panel. The ΔΔ Cp method was used to calculate fold change of gene expression [26]. For all measurements of gene expression, the FDR was controlled using the Benjamini-Hochberg procedure to adjust for multiple comparisons [20, 21]. Results were considered statistically significant if $p < 0.05$.

Results

Baseline and 35-wk RT characteristics of the subjects in the metabolomics study can be seen in Table 1. After 35 weeks of RT, a paired t-test demonstrated subjects increased appendicular lean mass (0.5 kg); consequently, there was a significant increase in SMI after RT (0.2 kg/m^2, Table 1). No changes in habitual intake of energy, carbohydrates, fat, vitamins, or minerals were found throughout RT. When the nutrients from the WPC80 shakes were excluded from the dietary analysis, there were no significant changes in total protein intake or individual amino acid intake. Therefore, we conclude that subjects maintained their habitual diet throughout the RT intervention. By calculating Pearson's correlation, we observed that SMI was not associated with dietary intake at baseline or after RT (**Table 3**).

Table 3. Association between skeletal muscle index (SMI) and dietary intake in older adults at baseline and after resistance exercise training (RT) [1]

	Baseline		RT	
	r	[2]p-value	r	[2]p-value
Total energy	0.27	0.99	0.40	0.71
Total protein	0.14	0.99	0.22	0.71
Total carbohydrate	0.36	0.99	-0.10	0.81
Total fat	0.17	0.99	0.55	0.71

[1] The Pearson's correlation, r, between dietary intake and SMI was calculated at baseline and after RT. n = 19 older adults.
[2] P-value after adjusting for multiple comparisons using the false discovery rate.

Metabolic pathways significantly associated with skeletal muscle index (SMI)

Using pathway topology analysis, MetaboAnalyst identified metabolic pathways that were associated with SMI at baseline and pathways associated with SMI after RT. The most impactful SMI-associated pathways were the same at baseline and after RT. Therefore, we grouped metabolite measurements from baseline and RT together and searched for pathways that were associated with SMI. MetaboAnalyst identified fifteen pathways that contained metabolites that were strongly associated with SMI (**Figure 2**, **Table 4**). Two of these pathways, *Valine, leucine, and isoleucine biosynthesis* and *Phenylalanine, tyrosine and tryptophan biosynthesis*, do not occur in humans and therefore, they are not relevant in this biological context. *Arginine and proline metabolism* was identified as the metabolic pathway that was most strongly associated with SMI, with a high impact and a high number of matches (Figure 2). The *Arginine and proline metabolism* pathway contains 77 metabolites, of which 20 were measured in the serum samples. MetaboAnalyst also calculated the Pearson's correlation between SMI and each individual metabolite, and seven of the 20 measured metabolites in the *Arginine and proline metabolism* pathway were individually associated with SMI ($p < 0.05$, FDR-corrected, **Figure 3**). Six of these seven metabolites (i.e., creatinine, glutamate, 1-pyrroline-2-carboxylate, proline, pyrroline-5-carboxylate, and l-4-hydroxy-glutamate semialdehyde) were positively associated with SMI and one metabolite (i.e., creatine) was negatively associated with SMI.

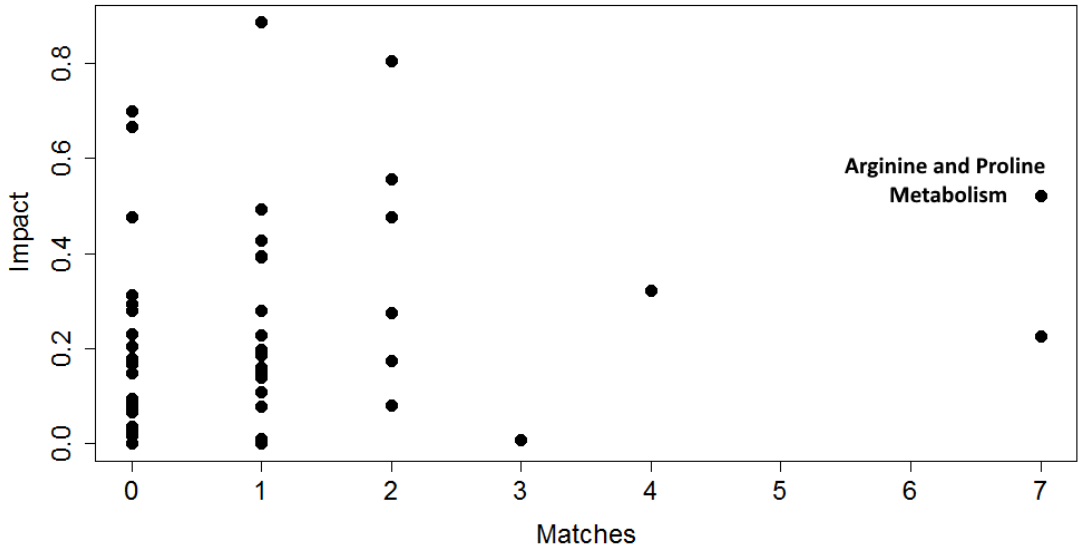

Figure 2: Impact and number of matches for each metabolic pathway associated with skeletal muscle index (SMI). The Pathway Analysis function of MetaboAnalyst was used to determine which biochemical pathways contained metabolites whose concentrations were associated with skeletal muscle index. Matches represent the number of metabolites in each pathway that were individually associated with SMI ($p > 0.05$, FDR-corrected). Impact represents how important the measured metabolites are to the entire pathway and is calculated using the relative betweenness centrality. *Arginine and proline metabolism* was the metabolic pathway with the highest combined matches and impact. SMI, skeletal muscle index.

Table 4. Metabolic pathways identified by MetaboAnalyst to contain serum metabolites associated with skeletal muscle index (SMI) [1]

Pathway	[2]Total Compounds	[3]Hits	[4]Matches	[5]Impact
Arginine and proline metabolism	77	20	7	0.52
Valine, leucine and isoleucine degradation	40	7	2	0.08
Valine, leucine and isoleucine biosynthesis	27	7	2	0.18
Aminoacyl-tRNA biosynthesis	75	19	7	0.23
Pyrimidine metabolism	60	14	2	0.27
Tryptophan metabolism	79	9	1	0.28
Phenylalanine, tyrosine and tryptophan biosynthesis	27	4	2	0.08
Porphyrin and chlorophyll metabolism	104	4	1	0.01
Linoleic acid metabolism	15	5	1	0.89
Riboflavin metabolism	21	2	1	0.15
Glutathione metabolism	38	7	1	0.08
Nitrogen metabolism	39	12	3	0.01
Butanoate metabolism	40	6	1	0.16
Sulfur metabolism	18	3	0	0.29
Sphingolipid metabolism	25	1	0	0.00

[1] Analysis was conducted using both baseline and RT measurements. n = 19 older adults. RT, resistance exercise training; SMI, skeletal muscle index.
[2] Total compounds, the number of compounds in the pathway
[3] Hits, the number of compounds in the pathway that were measured in serum
[4] Matches, the number of compounds in the pathway that were individually associated with SMI ($p > 0.05$)
[5] Impact, the importance of the hits in the pathway, based on the number of hits and their centrality in the metabolic network

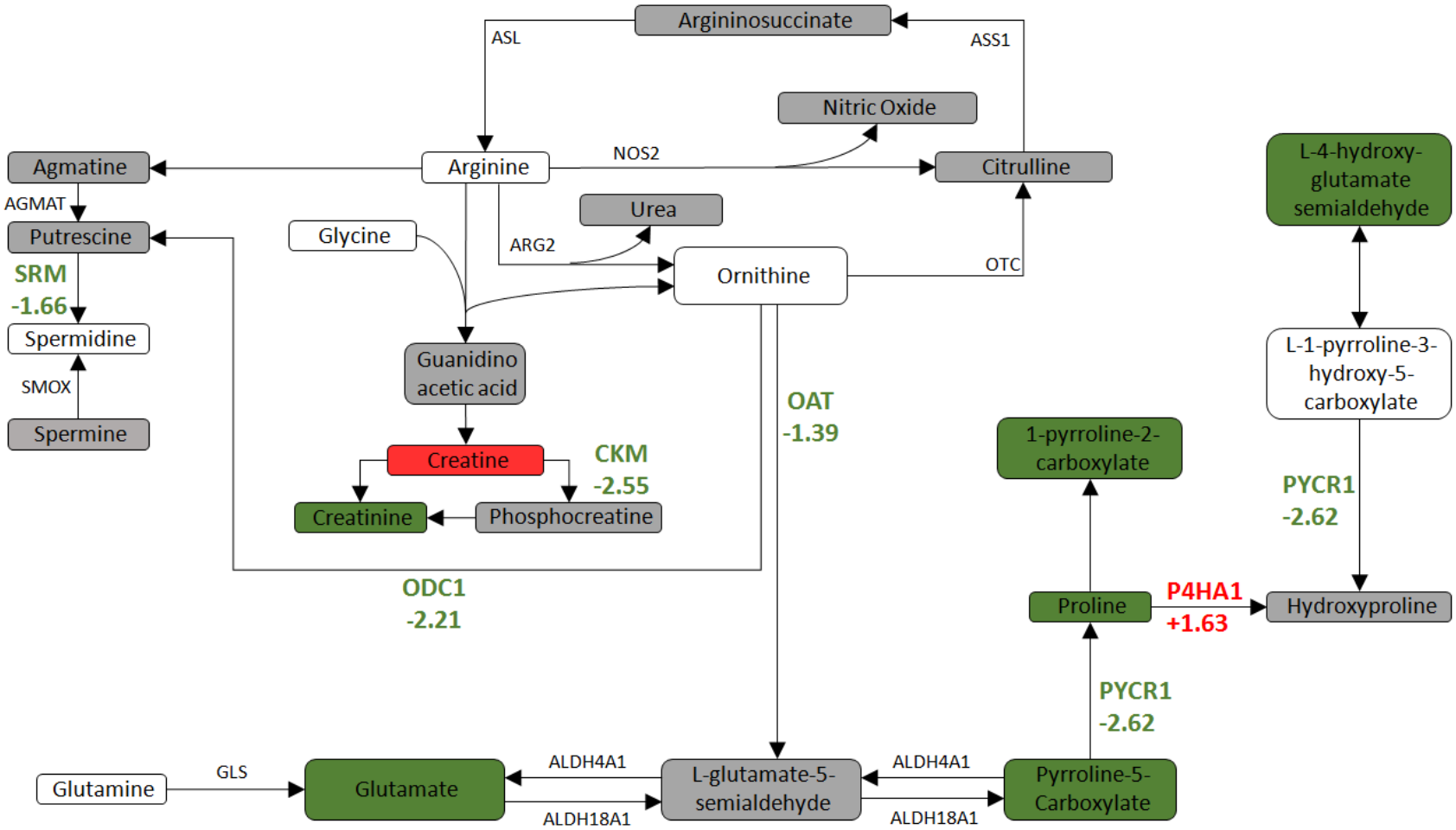
Argininosuccinate
ASL
ASS1
Nitric Oxide
Agmatine
Arginine
NOS2
Citrulline
L-4-hydroxy-glutamate semialdehyde
AGMAT
Urea
Glycine
ARG2
Putrescine
Ornithine
OTC
SRM
-1.66
Spermidine
Guanidino acetic acid
L-1-pyrroline-3-hydroxy-5-carboxylate
SMOX
OAT
-1.39
1-pyrroline-2-carboxylate
Spermine
Creatine
CKM
-2.55
PYCR1
-2.62
Creatinine
Phosphocreatine
ODC1
-2.21
Proline
P4HA1
+1.63
Hydroxyproline
PYCR1
-2.62
Glutamine
GLS
Glutamate
ALDH4A1
ALDH18A1
L-glutamate-5-semialdehyde
ALDH4A1
ALDH18A1
Pyrroline-5-Carboxylate

Figure 3: *Arginine and proline metabolism* was identified by MetaboAnalyst as the biochemical pathway most closely associated with skeletal muscle index (SMI) in the serum metabolites of 19 older adults (global test, $p = 0.0003$, FDR-corrected). MetaboAnalyst calculated the correlation of each individual metabolite with SMI (Pearson's r, $p < 0.05$, FDR-corrected). Compounds were positively associated (green box), negatively associated (red box), or not associated (white box) with SMI, or not measured (gray box) in our dataset. Further, inflammation induced by TNF-α, in our *in vitro* model, decreased (green print), increased (red print), or did not affect (black print) the gene expression of key arginine and proline metabolic enzymes that were measured (fold change below the corresponding enzyme). Paired t-tests were used to identify significant differences between treated and control cells ($p < 0.05$, FDR-corrected). *AGMAT*, agmatinase; *ALDH4A1*, pyrroline-5-carboxylate dehydrogenase; *ALDH18A1*, pyrroline-5-carboxylate synthase; *ARG2*, arginase 2; *ASL*, argininosuccinate lyase; *ASS1*, argininosuccinate synthase; *CKM*, creatine kinase, muscle; *GLS*, glutaminase; FDR, false discovery rate; *NOS2*, nitric oxide synthase 2 (inducible); *OAT*, ornithine aminotransferase; *ODC1*, ornithine decarboxylase 1; *OTC*, ornithine carbamoyltransferase; *PYCR1*, pyrroline-5-carboxylate reductase 1; *P4HA1*, prolyl 4-hydroxylase subunit alpha 1; SMI, skeletal muscle index; *SMOX*, spermine oxidase; *SRM*, spermidine synthase.

Arginine metabolism and skeletal muscle

To better understand the relationship between skeletal muscle health and arginine and proline metabolism, we measured the gene expression of the arginine transporters, cationic amino acid transporters 1 and 2 (*CAT-1* and *CAT-2*, respectively), in younger (21-39 y, n = 6 males and 5 females) and older (68-80 y, n = 4 males and 6 females) adult skeletal muscle biopsies collected in the follow-up experiment. A two-factor ANOVA showed that there were no age- or sex-related differences in the gene expression of *CAT-1* and *CAT-2* in the skeletal muscle tissue of the younger and older adults after the FDR correction (**Figure 4**).

Other studies have previously reported a link between *CAT-2* gene expression and inflammation in endothelial cells [27, 28]. Additionally, we have previously observed elevated markers of skeletal muscle inflammation in older adults [29], as well as clear evidence of heightened skeletal muscle inflammation susceptibility (MuIS) with advancing age that manifests both in muscle tissue and in isolated myogenic cells *in vitro* [30]. Therefore, we determined whether these older (vs. younger) adults had heightened MuIS. In a subset of subjects, a two-factor ANOVA on age and sex demonstrated that there were no differences between younger and older subjects in the gene expression of the inflammatory markers *TNF-α*, *STAT3*, *NFKB1*, *IL-6* (Figure 4), or the IL-6 receptors (*IL-6R* and *GP130*).

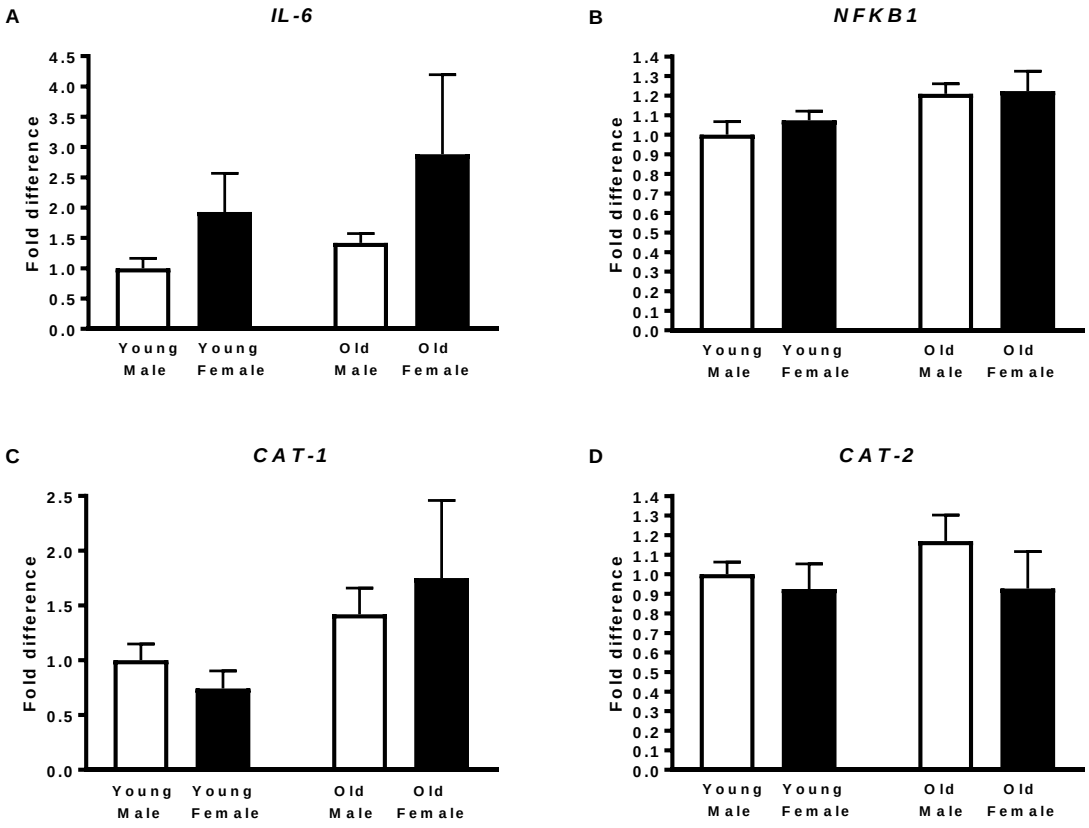

Figure 4: Gene expression of inflammatory signals (A) *IL*-6 and (B) *NFKB1*, and the arginine transporters (C) *CAT-1* and (D) *CAT-2* in human skeletal muscle tissue from young (n = 11) and old (n = 10) adults. Means ± SEM. A two-factor ANOVA was used to identify significant effects of age and sex ($p < 0.05$, FDR-corrected). Values were normalized to *TBP* gene expression. All values are expressed relative to gene expression in young males. *CAT*, cationic amino acid transporter; FDR, false discovery rate, *NFKB1*, nuclear factor kappa B; *TBP*, TATA-box binding protein.

Although we were likely underpowered, after adjusting for the FDR, to observe differences in MuIS in our follow-up cohort, the older adults did not appear to have heightened MuIS, based on a targeted gene expression profile. Therefore, we used a primary cell culture model to further investigate the effects of inflammation on arginine transport in differentiated primary human myoblasts/myotubes. Cells from young adults were used for these experiments to isolate the effects of inflammation from other age-related effects. Paired t-tests showed that treating differentiated primary myoblasts/myotubes from young male (n = 6) and young female (n = 5) adults with TNF-α induced a 32-fold increase in the gene expression of *IL-6* in males ($p < 0.001$, FDR-corrected) and a 16-fold increase in females ($p < 0.001$, FDR-corrected, **Figure 5**). TNF-α also induced a 3.1-fold increase in the gene expression of *NFKB1* in males ($p < 0.001$, FDR-corrected, Figure 5) and a 4.3-fold increase in females ($p < 0.001$, FDR-corrected, Figure 5). Although *CAT-1* gene expression changed in male myoblasts/myotubes incubated in TNF-α ($p = 0.045$, FDR-corrected), the -1.1 fold-change was not biologically meaningful. There was no change in *CAT-1* gene expression in female cultures treated with TNF-α. However, the expression of *CAT-2* increased 11-fold in males ($p = 0.0023$, FDR-corrected) and 23-fold in females ($p = 0.001$, FDR-corrected) following 48 hour TNF-α treatment (Figure 5).

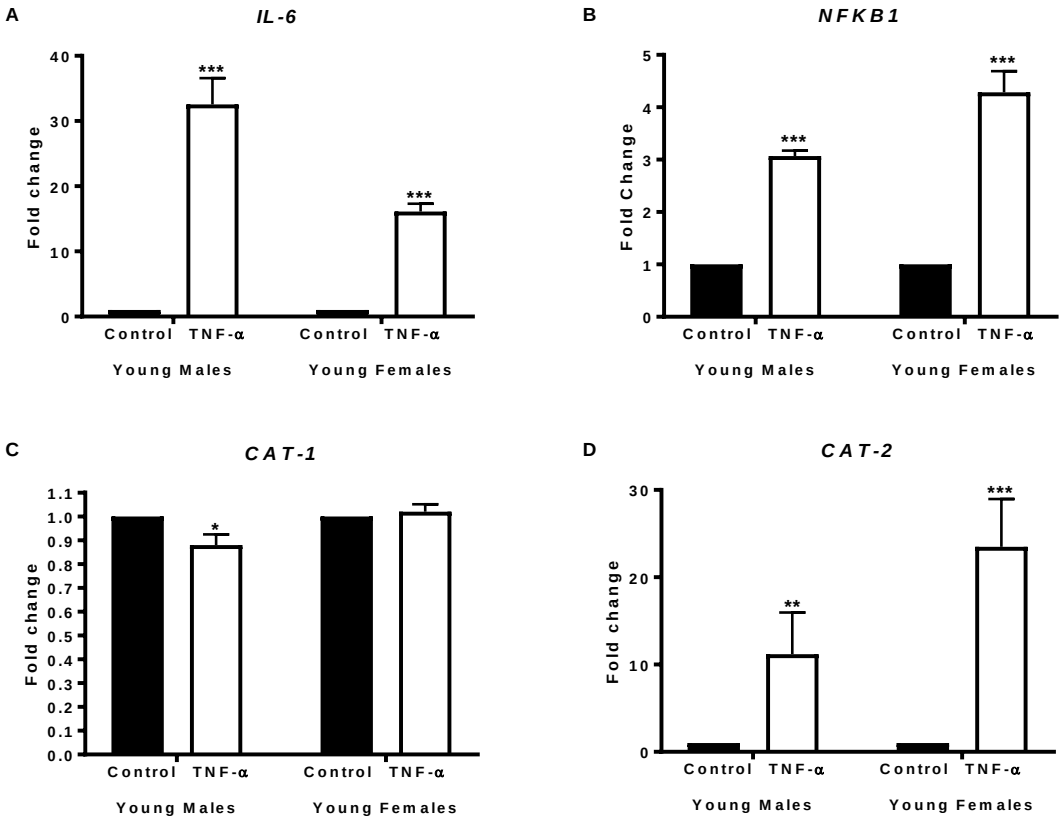

Figure 5: Gene expression of *IL-6*, *NFKB1*, and arginine transporters *CAT-1* and *CAT-2* in human primary skeletal muscle cells that were incubated in TNF-α to induce inflammation. Means ± SEM. Values were normalized to *TBP* gene expression (females, n=5) or *18S* gene expression (males, n=6) and are expressed relative to same-sex controls. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, significantly different from untreated controls (paired t-test) after FDR-correction. *NFKB1*, nuclear factor kappa B subunit 1; *TBP*, TATA-box binding protein.

The magnitude of the *CAT-2* response in the female cells treated with TNF-α led us to test the effects of inflammation on the expression of key metabolic genes important for arginine and proline metabolism in the female cells. Of the sixteen genes analyzed, inflammation induced a decrease in the expression of five genes (ornithine decarboxylase 1, pyrroline-5-carboxylate reductase 1, spermidine synthase, creatine kinase, and ornithine aminotransferase) and an increase in the expression of one gene (prolyl-4-hydroxylase subunit alpha 1) (**Table 5,** Figure 3).

Table 5. Changes in the gene expression of arginine metabolic enzymes induced by TNF-α *in vitro* in human primary skeletal muscle cells

Gene Symbol	Gene Name	p-value †	Fold-change
ODC1	ornithine decarboxylase 1	0.0010*	-2.21
PYCR1	pyrroline-5-carboxylate reductase 1	0.0016*	-2.62
SRM	spermidine synthase	0.0096*	-1.66
CKM	creatine kinase, muscle	0.0108*	-2.55
P4HA1	prolyl 4-hydroxylase subunit alpha 1	0.0165*	1.63
OAT	ornithine aminotransferase	0.0200*	-1.39
GLS	glutaminase	0.0609	-1.34
ALDH18A1	pyrroline-5-carboxylate synthase	0.0609	-1.28
ASS1	argininosuccinate synthase	0.0774	-1.49
NOS2	nitric oxide synthase 2 (inducible)	0.0797	-1.53
OTC	ornithine carbamoyltransferase	0.0797	-1.53
ALDH4A1	pyrroline-5-carboxylate dehydrogenase	0.2049	-1.37
SMOX	spermine oxidase	0.2783	-1.31
ARG2	arginase 2	0.4853	1.04
ASL	argininosuccinate lyase	0.7134	-1.06
AGMAT	agmatinase	0.7134	-1.06

Cultured human primary skeletal muscle cells from young adult females were incubated in TNF-α to induce inflammation. qPCR was used to measure changes in the gene expression of enzymes governing arginine metabolism. n = cells from 5 young females grown in triplicate *in vitro*.

† P-value after adjusting for multiple comparisons using the false discovery rate. *, $p < 0.05$, significantly different from paired control (paired t-test).

Discussion

This study is the first to identify an association between *Arginine and proline metabolism* and a marker of skeletal muscle health, SMI, in older adults. While other key amino acid metabolism pathways (e.g., *Valine, leucine, and isoleucine degradation*) were also associated with SMI, *Arginine and proline metabolism* was the most strongly associated pathway with the largest impact. Although possible, it is unlikely that the observed association is attributed to consumption of arginine- and proline-rich foods, because the serum used for the metabolite analysis was collected after an overnight fast, and habitual dietary intake of arginine, proline, or a substrate used for the synthesis of these amino acids was not associated with SMI at baseline or after RT (data not shown). Based on our follow-up analyses, we can speculate whether the observed association between arginine and proline metabolism and SMI is due to low-grade inflammation and heightened MuIS observed with skeletal muscle atrophy in older adults.

Arginine is a conditionally-essential amino acid that is synthesized from other metabolites in the body. The majority of endogenous arginine synthesis in human adults occurs when citrulline travels from the small intestine to the kidney, where it is converted into arginine by argininosuccinate synthase [31]. Glutamine, glutamate, and proline serve as precursors for citrulline [31]. Healthy adults have the capacity to synthesize sufficient arginine to satisfy metabolic requirements [32]. In the current study, subjects with lower SMI also displayed diminished circulating glutamate and proline, potentially indicating an insufficient precursor supply for citrulline production and thus insufficient intestinal-renal arginine synthesis in older adults with lower

muscle mass. It is possible that alterations in arginine metabolism and availability affect SMI by altering muscle protein synthesis. Arginine activates the mechanistic target of rapamycin (mTOR) signaling pathway, increasing phosphorylation of the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and the fractional rate of skeletal muscle protein synthesis [33]. Future research is necessary to determine whether the relationship between arginine metabolism and SMI is linked to mTOR activity.

Studies demonstrate that supplements containing arginine are beneficial for maintaining skeletal muscle mass or performance in aging or chronically ill individuals who likely have elevated inflammation [34-36]. Older women who received an 8.5 g mixture of arginine, β-hydroxy β-methylbutyrate (HMB), and lysine for 12 weeks had 20% higher whole-body protein synthesis compared to patients who consumed an isocaloric maltodextrin placebo, although there was no significant difference between groups in net protein gain [35]. The older women also had increased leg strength and functionality [35]. Additionally, adults with HIV who consumed a supplement consisting of arginine, HMB, and glutamine daily for eight weeks increased their lean body mass (LBM), while subjects who received a maltodextrin placebo lost LBM [34]. Adults with cancer cachexia who consumed a similar supplement (as the adults in the HIV study) for 24 weeks increased their fat-free mass over 24 weeks [36]. Despite the positive effects of arginine on skeletal muscle in older women and chronically ill patients, arginine supplementation did not improve performance in healthy athletes [37-40]. These differential responses to supplements containing arginine, among various populations, suggest alterations in

arginine synthesis and/or metabolism that would make arginine an essential amino acid in some populations, including older adults with declining skeletal muscle mass.

Supplements containing arginine were beneficial to populations that have elevated inflammation [41], which is a key factor in skeletal muscle regeneration and deterioration. The acute inflammation that occurs after exercise is vital for muscle regeneration and thought to be responsible for the beneficial effects of exercise on whole-body health [42]. In contrast, chronic low-grade inflammation is associated with aging [43] and metabolic disease [44], negatively associated with physical activity [45], and causes skeletal muscle to lose myofibers and gain fibrotic and adipose tissues. Circulating levels of the proinflammatory cytokine TNF-α, a signaling molecule and initiator of inflammation [46], are positively associated with diminished muscle strength [47, 48] and mortality [49, 50] in older adults. TNF-α activates the transcription factor NF-κB, which is associated with multiple skeletal muscle disorders, including Duchenne muscular dystrophy, inflammatory myopathies, cancer cachexia, and disuse atrophy [51].

Given the association between older adults, inflammation, and sarcopenia, we sought to determine whether the relationship between skeletal muscle and arginine metabolism involved inflammation, specifically TNF-α. TNF-α regulates arginine metabolism in rat neuronal cells, rat cardiac myocytes, and human vascular smooth muscle [28] by increasing gene expression of the cationic amino acid transporter (*CAT-2*). The CAT isoforms (CAT-1, CAT-2A, and CAT-2B) allow arginine, lysine, and ornithine to pass through the plasma membrane. TNF-α increases the gene expression of *CAT-2* in human endothelial cells by activating the NF-κB signaling

pathway [28], and NF-κB DNA-binding activity is increased by low arginine availability [52]. We predicted older adults would have an elevated proinflammatory gene expression profile and elevated arginine transporter expression compared to younger adults. Surprisingly, young and old subjects had similar levels of inflammatory and *CAT-1* and *CAT-2* gene expression, potentially due to the relatively active, healthy older adults in the follow-up study. It is also possible that we were underpowered, after adjusting for the FDR, to measure differences in the gene expression of the arginine transporters. We also predicted that TNF-α increases *CAT-2* gene expression in human skeletal muscle cells, increasing arginine uptake. We observed a robust increase in *CAT-2* gene expression in primary myoblast/myotube cultures from males and females following incubation with TNF-α, a response that has been previously reported in other cell lines [27, 28], but not in skeletal muscle cells. Although we do not know if arginine uptake was altered, the increased expression of this arginine transporter may be a signal of increased demand for arginine in an inflamed state. Intriguingly, it has been demonstrated that arginine is important for attenuating NF-κB activity in keratinocytes [52]. We demonstrated that *NFKB1* expression is elevated in our culture model, further supporting a potential role of arginine during heightened inflammation.

Forty-eight hours of inflammation affected key metabolic pathways for arginine and proline metabolism, including decreased polyamine production and proline synthesis. The timing and duration of this inflammation may have been critical to the observed effects. Decreased expression of *ODC1* and *SRM* with TNF-α treatment could be an indication of disrupted skeletal muscle regenerative and/or

atrophic mechanisms through disruption of polyamine synthesis. Polyamines such as putrescine and spermidine are important for the regulation of cell proliferation and delay of myoblast differentiation [53]. Spermine oxidase (*SMOX*) converts the polyamine spermine into spermidine and regulates genes that control muscle atrophy [54]. Inflammation also altered the expression of proline and hydroxyproline synthesis genes (*OAT*, *PYCR1*, and *P4HA1).* Proline and hydroxyproline comprise 25% of the amino acid residues of collagen, which is critical for wound healing [55]. Lastly, *CKM* gene expression was reduced with inflammation, an indication that myoblast fusion is disrupted in our model [56]. Lower gene expression of key metabolic enzymes with inflammation (*in vitro)* is consistent with lower levels of the metabolic products from these enzymatic reactions that we observed in older individuals with low SMI (*in vivo,* Figure 3).

Limitations

Because metabolites were measured in serum and not in the muscle, it is not possible to definitively distinguish muscle metabolic changes from metabolic changes that occur in other organ systems. However, measuring serum metabolites makes it possible to identify potential inter-organ cross-talk. In addition, the nature of metabolomics makes it difficult to establish causation. However, the corroborating results from the metabolomics and transcriptomics studies allow us to generate new hypotheses and directions for future research to understand metabolic changes in arginine and proline metabolism associated with sarcopenia.

Conclusions

In conclusion, we determined that the *Arginine and proline metabolism* pathway was most strongly associated with skeletal muscle mass in older adults; several metabolites of arginine and proline metabolism were observed to be lower in individuals with lower muscle mass. Further, we identified differential gene expression of arginine and proline metabolic enzymes with inflammation, results that corroborated the metabolomics study and help explain the relationship between arginine and proline metabolism and SMI with aging. While future research is necessary to fully elucidate the relationship between arginine and proline metabolism and skeletal muscle mass with aging, the present findings suggest therapies to manipulate the availability of arginine may improve skeletal muscle health in adults with chronic inflammation.

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The authors' responsibilities were as follows: Diwakar Gupta, Anna Thalacker-Mercer designed research; Diwakar Gupta, Jamie Elaine Blum, Xiaojing Liu, Heather Bartlett Roman conducted research; Erica Bender, Marcas Bamman, Jason Locasale provided essential materials and instruments; Diwakar Gupta, Irina Gaynanova, Anna Thalacker-Mercer analyzed data; Diwakar Gupta, Anna Thalacker-Mercer wrote paper; Anna Thalacker-Mercer had primary responsibility for final content. All authors read and approved the final manuscript.

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CHAPTER 3

SUMMARY AND FUTURE DIRECTIONS

Summary

The aim of this study was to identify circulating factors that regulate skeletal muscle mass in older adults, in order to identify metabolic disturbances that are associated with age-related skeletal muscle atrophy (i.e. sarcopenia) with the goal to identify potential therapeutic targets to attenuate sarcopenia.

We identified, using the serum of older adults, that the *Arginine and Proline Metabolism* pathway was the metabolic pathway most strongly associated with skeletal muscle index (SMI) and the pathway that had the greatest impact. SMI is positively correlated with circulating levels of creatinine, glutamate, pyrroline-5-carboxylate, proline, 1-pyrroline-2-carboxylate, and L-4-hydroxy-glutamate semialdehyde, and negatively correlated with circulating creatine. Upon initial examination of the current literature, the relationship between arginine and SMI in older adults was unclear; however, we identified that disruption of arginine metabolism may be driven by inflammation: in clinical trials, supplements containing arginine improve skeletal muscle health, but only in populations suspected to have elevated inflammation [1-3].

To investigate the relationship between inflammation and arginine in skeletal muscle, we measured gene expression in the skeletal muscle of younger and older adults. We hypothesized that older adults would have higher inflammatory signaling and altered expression of the cationic amino acid transporter *CAT-2*. Surprisingly, there were no differences between younger and older adults in inflammatory signaling

or in the gene expression of *CAT-1* or *CAT-2.* We believe that because younger and older adults had similar levels of inflammation, they also had similar *CAT-1* and *CAT-2* gene expression.

We turned to a primary human muscle cell model where we could induce inflammation to determine the relationship between inflammation and arginine *in vitro.* Incubation in the inflammatory cytokine TNF-α for 48 hours increased the gene expression of *CAT-2* 11-fold in males and 23-fold in females, demonstrating a link between inflammation and arginine metabolism in human skeletal muscle cells. Additionally, we measured the expression of genes that regulate arginine metabolism in differentiated muscle cells (myotubes) from five young females. Inflammation decreased the expression of genes regulating the synthesis of polyamines, which are correlated with exercise in rat skeletal muscle [4] and regulate cell proliferation and differentiation [5]. Gene expression of ornithine decarboxylase 1, which increases myoblast proliferation and delays differentiation [6], and spermidine synthase was reduced. A reduction in spermine oxidase, the enzyme that breaks down spermine into spermidine, causes muscle atrophy in mice [7]. In addition, TNF-α changed the expression of genes that regulate proline and hydroxyproline production, including a decrease in pyrroline-5-carboxylate reductase 1, an increase in prolyl 4-hydroxylase subunit alpha 1, and a reduction in ornithine aminotransferase. Proline and hydroxyproline are the most common amino acid residues in collagen, which is important for wound healing. These inflammation-induced changes in gene expression suggest that arginine metabolism may regulate skeletal muscle mass in older adults through changes in the metabolism of polyamines and proline metabolism.

Future research

Although we observed increases in the gene expression of *CAT-2* in response to TNF-α treatment, we do not know if there is a concomitant increase in CAT-2 protein, nor do we know if arginine transport into skeletal muscle is increased by inflammation. As a future direction, CAT-2 protein expression and arginine uptake need to be measured in human myotubes to confirm that inflammation increases arginine transport in skeletal muscle cells.

Another question that warrants further investigation is how arginine is catabolized in inflamed human myotubes. Our research indicates that inflammation increases *CAT-2* gene expression, likely increasing arginine influx into myotubes. At the same time, inflammation decreases the amount of arginine used for polyamine synthesis and proline synthesis. The cellular need for increasing arginine during the inflammatory state remains unclear. Arginase and inducible nitric oxide synthase (iNOS) are two important pathways for arginine catabolism; however, arginase and iNOS were not differentially expressed after 48 hours of inflammation. It is possible that arginase and iNOS gene expression does change, but this change is not apparent at the 48-hour time-point. Alternatively, arginase or iNOS activity may be increased by inflammation through post-translational modifications. In future research, I would propose to culture myotubes in media containing arginine comprised of stable, isotopically labeled carbon atoms. Metabolic products formed from labeled arginine will contain these labeled carbon atoms, making it possible to precisely measure these metabolic products using mass spectrometry. Consequently, it will be possible to

determine how inflammation changes the relative distribution of arginine metabolic products.

Our research demonstrates that inflammation results in increased gene expression of *CAT-2* and changes in arginine metabolism. However, it is unclear if the changes in arginine metabolism induced by TNF-α are mediated by *CAT-2* and altered arginine supply, or by an independent inflammatory mechanism unrelated to arginine. To determine if manipulating the availability of arginine can improve skeletal muscle health, it is important to distinguish the effects of inflammation from the effects of changes in arginine transport. This question can be investigated by manipulating the expression of *CAT-2*. If knocking down *CAT-2* prevents TNF-α from causing the changes in the gene expression that we observed, then the effects of TNF-α are mediated by *CAT-2* and alterations in arginine concentration.

Further research into the effects of inflammation on whole-body arginine metabolism is also needed to translate these results into effective therapies to preserve skeletal muscle mass. First-pass extraction of dietary arginine [8] and endogenous arginine synthesis [9] determine the amount of arginine in circulation, and therefore, the amount of arginine available to skeletal muscle. It is unknown how these processes are altered by factors such as systemic or local inflammation, or by age or sex. Studying these interactions will provide insight into the cross-talk that occurs between skeletal muscle and the rest of the body and to potential therapies to attenuate the age-related loss of skeletal muscle mass.

In conclusion, we sought to understand the relationship between sarcopenia and whole-body metabolism, and determined that skeletal muscle mass in older adults was most strongly associated with the *Arginine and proline metabolism* pathway. Individuals with lower muscle mass had lower levels of several metabolites of arginine and proline metabolism. Suspecting that inflammation played a role in the relationship between arginine and proline metabolism and skeletal muscle mass in aging adults, we measured key arginine and proline metabolic enzymes. We observed differential gene expression of arginine and proline metabolic enzymes with inflammation, results that corroborated the metabolomics study and suggest that increased inflammation may alter the demand for arginine in muscle. While further research is needed to elucidate the relationship between arginine and proline metabolism and skeletal muscle mass with aging, these findings suggest that manipulation of the availability of arginine may be an effective therapy to improve skeletal muscle health in adults with chronic inflammation.

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