A. **Title:** Skeletal muscle acute and chronic metabolic response to essential amino acid supplementation in hypertriglyceridemic older adults.

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I. (i) OSM included

   (ii) Abbreviations:

   3MC4 : Isovaleryl carnitine

   2MC4: 2-methyl-butyryl-carnitine

   2MC3 : Isubutyryl-carnitine

   2MC3OH : 3 Hydroxy-isobutyryl-carnitine

   3MC4OH : 3OH-isovaleryl-carnitine

   αKG : alpha ketoglutarate

   BCAA : Branch Chain Amino Acids

   C2 : Acetyl-carnitine

   C3 : Propionyl-carnitine

   C4 : Butyryl Carnitine

   C4OH : 3 Hydroxy-butyryl-carnitine

   C4DC : Succinyl-carnitine
C6: Hexanoyl Carnitine
C8: Octanoyl Carnitine
C10: Decanoyl Carnitine
C14: Myristoyl Carnitine
C18: Stearoyl Carnitine
CA: Citrate
EAA: Essential Amino Acids + Arginine
EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
FA: Fumarate
HFBA: Heptafluorobutyric acid
HPLC: High performance liquid chromatography
iCA: Isocitrate
LA: Lactate
MA: Malate
OA: Oxaloacetate
OBA: O-benzylhydroxylamine
PA: Pyruvate
SD: Standard Deviation
SA: Succinate
TG: Triglyceride
TCA: Tricarboxylic Acid

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(iv) Conflicts of Interest

Marquis : No conflicts of interest
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Abstract

Background: Supplementation with essential amino acids + arginine (EAAs) is a promising nutritional approach to decrease plasma triglyceride concentrations, which are an independent risk factor for coronary heart disease.

Objective: The objective of this study was to examine the effects of eight weeks of EAA supplementation on skeletal muscle basal metabolite concentrations and changes in metabolic response to acute EAA intake, emphasizing mitochondrial metabolism, in adults with elevated triglycerides, to better understand the mechanisms of lowering plasma triglycerides.

Methods: Older adults with elevated plasma triglyceride concentrations were given 22 g of EAAs to ingest acutely before and after an eight-week EAA supplementation period. Skeletal muscle biopsies were collected before and after acute EAA intake, both pre- and post-supplementation (4 biopsies), and targeted metabolomic analyses of organic acids and acylcarnitines were conducted on the specimens.

Results: Acute EAA intake resulted in increased skeletal muscle acylcarnitine concentrations associated with oxidative catabolism of the supplement components, with the largest increases found in acylcarnitines of BCAA oxidative catabolism including isovaleryl-carnitine (2200%) and 2-methylbutyryl-carnitine (2400%). The chronic EAA supplementation resulted in a 19% decrease in plasma triglycerides along with accumulation of long-chain acylcarnitines myristoyl- (90%) and stearoyl- (120%) carnitine in skeletal muscle, and increases in succinyl-carnitine (250%) and late-stage tricarboxylic acid cycle intermediates fumarate (44%) and malate (110%).

Conclusion: EAA shows promise as an approach for moderate reduction in plasma triglycerides. Changes in skeletal muscle metabolites suggest incomplete fatty acid oxidation and increased anaplerosis suggesting a potential bottleneck in fatty acid metabolism.

Keywords: Acylcarnitines; Essential Amino Acids; TCA Intermediates; Targeted Metabolomics; Triglycerides
Introduction

Hypertriglyceridemia is a significant independent risk factor for coronary heart disease, which is the single largest killer of men and women in the United States (1). Hypertriglyceridemia prevalence increases with age and is associated with obesity, renal disease, and diabetes (2). The treatment of elevated triglycerides (TG) is included in the Adult Treatment Panel III (ATP III) of the National Cholesterol Education Program (3, 4). Revising dietary macronutrient intake is a primary strategy for reducing TG. Although limiting intake of saturated and trans fatty acids, and cholesterol, is widely advocated to lower TG, it is also well established that higher carbohydrate diets increase plasma TG concentrations (5), and low-carbohydrate diets have been found to reduce plasma TGs more than low-calorie, low-fat diets do (6). As a reduction in dietary carbohydrate is often accompanied by an increase in protein intake, and the independent impact of these two factors on plasma TG concentrations may be difficult to differentiate, it is interesting to note that dietary supplementation of an *ad libitum* diet with protein results in a lowering of plasma TG (7). The influence of amino acid intake on plasma lipid concentrations is largely unknown and is an active area of research (8-10) to better isolate the effects of protein intake on lipid levels.

In our previous studies in older adults with impaired glucose tolerance, we observed decreased circulating and tissue TG concentrations after supplementing the diet with a small quantity of essential amino acids (EAA) + arginine (~22 g/day) between meals for 16 weeks (9). In another study, we found that a combination supplement consisting of EAAs with whey protein and phytosterols reduced plasma cholesterol and TG levels in overweight people with mild hyperlipidemia (10). Others have found that obese people exhibit alterations in skeletal muscle metabolism of amino acids and fatty acids relative to lean people (11), with evidence of increased anaplerosis and limited fatty acid oxidative flux. A recent study reported the co-existence of elevated
short/medium-chain acylcarnitines and reduced branched-chain ketoacids and tricarboxylic acid (TCA) cycle intermediates in skeletal muscle from people with insulin resistance, suggesting that impaired skeletal muscle metabolism of branched-chain amino acids (BCAAs) and lipids may play a role in the mechanisms underlying reduced insulin sensitivity (12).

This important link between fatty acid and EAA oxidative metabolism is tied to mitochondrial function where the oxidative fate of these classes of molecules ultimately resides through β-oxidation and entrance into the TCA cycle (13, 14). In fact, there is in vitro evidence for a “branched-chain” carnitine-acylcarnitine translocase in the mitochondria implicated in BCAA oxidative metabolism (15). Increasing oxidative flux through BCAA pathways within cultured skeletal muscle cells has been shown to effect a significant increase in fatty acid oxidation, with the implication that obstructions to BCAA oxidation could limit skeletal muscle lipid turnover (12). To better understand this relationship and to inform future nutritional interventions in hypertriglyceridemia, we investigated the effects of EAA supplementation on mitochondrial metabolism.

Materials and Methods

Reagents and Chemicals

Liquid chromatography (LC) mobile phases consisting of HPLC-grade acetonitrile and modifiers formic acid and heptafluorobutyric acid (HFBA) were purchased from Sigma Aldrich, while water was generated using an in-house Milli-Q water purification system. Other chemicals, including o-benzylhydroxylamine (OBHA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), ethyl acetate, and methanol, were also purchased from Sigma Aldrich. Internal standards for targeted metabolomics including sodium lactate $^{13}$C$_3$, citric D$_4$(2,2,4,4) acid, fumaric $^{13}$C$_4$ acid, sodium pyruvate $^{13}$C$_3$, α-ketoglutaric D$_6$ acid, butyryl-l-carnitine D$_3$, propionyl-l-carnitine D$_3$, acetyl-l-
carnitine D₃, decanoyl-l-carnitine D₃, hexanoyl-l-carnitine D₃, isovaleryl-l-carnitine D₉, octadecanoyl-l-carnitine D₃, octanoyl-l-carnitine D₃, and tetradecanoyl-l-carnitine D₃ were purchased from Isotec (Sigma Aldrich, Milwaukee, WI), while succinic D₆ acid and disodium-2-hydroxy-glutarate D₃(2,3,3) were purchased from CDN Isotopes (Pointe-Claire, QC).

Subject Information and Experimental Protocol.

A total of six older [69 ± 4 (SD) years, BMI 35 ± 9] volunteers (4 females, 2 males) with elevated plasma triglycerides [2.3 ± 0.4 mmol/L] participated in an 8-week study period (Fig. 1). Exclusion criteria included diabetes, evidence of renal or liver disease, bleeding disorders, endocrine disease (excluding well-controlled hypothyroidism), and alcohol or drug abuse. Prior to beginning the study, the subjects were counseled to maintain their typical dietary intake and physical activity pattern. During the subjects’ study visits and in telephone calls between visits, they were asked about dietary changes and reminded to not make any changes. Before and after an 8-week EAA supplementation phase, subjects underwent an acute EAA intake experiment wherein they arrived after an overnight fast and a basal muscle biopsy was collected from m. vastus lateralis as described elsewhere (16), before consuming 1 g of an EAAs plus arginine supplement dissolved in water every 10 minutes for the next 3.5 h (22 g of EAAs plus arginine in total). The composition of the EAAs was as follows: 3.26% histidine, 8.57% isoleucine, 35.88% leucine, 17.08% lysine-HCl, 3.59% methionine, 4.65% phenylalanine, 9.57% threonine, 7.44% valine, 9.97% arginine. The EAA mixture was provided by Ajinomoto (Itasca, IL). Upon completion of the acute EAA intake, another muscle biopsy was taken. During the chronic supplementation phase, subjects took two 11 g doses daily in capsule form; one dose between breakfast and lunch, and the other dose between lunch and dinner. The subjects recorded their doses in a diary, and visited the hospital in order to receive a new supply of supplements every two weeks. See Table 2 for subject characteristics. The protocol was approved by
the Institutional Review Board at the University of Arkansas for Medical Sciences and informed consent was obtained from all subjects participating in the study.

Sample Preparation

Skeletal Muscle Preparation. A portion of the skeletal muscle biopsies was snap frozen immediately after collection and stored at -80°C until the study was complete. The samples were batch processed to minimize variation and were processed alongside a set of standard porcine muscle samples to assess the presence of experimental error. A more complete description of sample processing for the skeletal muscles is outlined elsewhere (17). Briefly, approximately 15–25 mg of frozen skeletal muscle was weighed and then transferred to a pre-chilled stainless steel homogenization tube loaded with silicon carbide sharp particles (Biospec Products, Bartlesville, OK). Pre-chilled 50:50 methanol:water extraction buffer was added to each tube at a ratio of 13 μL of buffer per mg of tissue and then each tube was spiked with a mixture of isotope labeled standards for organic acids and acylcarnitines (see supplementary Tables 1-2 for isotope standard details). Samples were then homogenized using a bead ruptor 24 (Omni International Inc., Kennesaw, GA) using two 30 s homogenization steps at 6.3 m/s with a subsequent dry ice cool-down step in between. Samples were centrifuged for 10 minutes at 14 kG at 4°C and the raw extract supernatant was transferred to a fresh tube and stored at -80°C until subsequent analysis.

Organic acid sample preparation. An aliquot of the raw skeletal muscle extract was derivatized as described elsewhere (18). Briefly, 100 μL of muscle extract was mixed with 50 μL of 1 M OBHA (in pyridine buffer, pH 5.1) followed by the addition of 50 μL 1 M EDC (in pyridine buffer, pH 5.1). The mixtures were shaken at room temperature for 1 h, then extracted with ethyl acetate three times. The
combined organic layers were dried by SpeedVac and then reconstituted in mobile phase (as described below) and transferred to a vial for chromatography measurements.

Acylcarnitine sample preparation. Full details of the acylcarnitine preparation are described elsewhere (19). Briefly, aliquots of the skeletal muscle raw extract were transferred to tubes, dried by SpeedVac, and reconstituted in 0.1% phosphoric acid. Acylcarnitines were then loaded onto Oasis MCX extraction cartridges (Waters Corp., Milford, MA), activated by methanol immediately prior to sample loading. Cartridges were washed twice with 0.1% phosphoric acid before samples were eluted with methanol. Eluates were dried by SpeedVac, reconstituted in mobile phase (as described below), and transferred to a vial for chromatography.
Chromatography Mass Spectrometry.

All LC-MS/MS was conducted using an ABSciex Qtrap 5500 mass spectrometer with an Eksigent Micro 200 LC and controlled by Analyst 1.6.3 software (Sciex Inc., Framingham, MA). Organic acid OBHA derivatives were separated using a 0.3x150-mm ChromXP C18EP column with a 3-μm particle size and 300-Å pore size (Eksigent, Sciex Inc.). Mobile phases for organic acid analysis consisted of Milli-Q water with 1% formic acid (A) and acetonitrile with 1% formic acid (B). The chromatographic elution program for organic acids was run with a flow rate of 25 μl/min and began by holding the mobile phase at 25% B for 30 s, followed by a linear gradient to 70% B for 5 minutes, then a 30 s linear gradient to 100% B where it was held for a final 30 s. Acylcarnitines were separated on a 0.5x150-mm Halo C18 column with a 2.7-μm particle size and a 90-Å pore size (Eksigent, Sciex Inc.). The mobile phases for acylcarnitine analysis consisted of Milli-Q water with 0.08% HFBA ion pairing agent (A) and acetonitrile (B). The elution program for acylcarnitines was run at 25 μL/min using a stepped gradient profile from 5% B to 95% B over 10 minutes (see Supplemental Tables 1 and 2 for more details). Mass spectrometry was conducted using multiple reaction monitoring (MRM) following the settings as described in the supplementary information. Sample concentrations were determined using MRM peak ratios with internal standards as described in the supplementary information.

Data Analysis

MRM peak areas were measured using Analyst 1.6.4 Software and MultiQuant and were visually inspected. Muscle sample masses and chromatographic peak areas of analytes and isotope standards were imported into R software for statistical analysis. Mass concentrations of analytes were calculated in terms of analyte mass per mg of muscle tissue. Grubb’s test for outliers (α =0.05) was performed on subjects’ metabolite concentration response to both the acute EAA challenge and the 8-
week EAA supplementation. 9 out of 576 metabolic responses measured were determined to be outliers by the Grubb’s test and removed from the analysis. Statistical analysis was conducted using paired t tests ($\alpha = 0.05$) to compare each subject’s basal concentrations for skeletal muscle metabolites before and after the 8-week supplementation period. Paired t tests were also conducted to compare skeletal muscle metabolite concentrations before and after the periods of acute EAA intake.

### Results

#### Plasma TG concentrations

After 8 weeks of EAA supplementation, fasted plasma TG concentrations were found to decrease by from $2.3 \pm 0.4$ mmol/L to $1.8 \pm 0.3$ mmol/L [19% ($p < 0.05$)]. This TG plasma decrease is consistent with those reported elsewhere (9, 10).

#### Short-chain acylcarnitines

The skeletal muscle acylcarnitine concentrations related to oxidative metabolism of EAAs yielded the most dramatic changes in response to EAA challenge (Fig. 2). Acylcarnitine metabolites related to oxidation of leucine and isoleucine, i.e., isovaleryl-carnitine (3MC4) and 2-methylbutyryl-carnitine (2MC4), increased by 2200% ($p < 0.04$) and 2400% ($p < 0.008$), respectively, in response to acute EAA intake (Fig. 3). 3-hydroxybutyryl-carnitine (C4OH), a metabolite of lysine metabolism, was also found to increase in response to acute EAA intake by 132% ($p < 0.04$). The metabolic intermediates of valine oxidation, isobutyryl-carnitine (2MC3, 136%, $p < 0.01$) and 3-hydroxy-isobutyryl-carnitine (2MC3OH, 95%, $p < 0.05$), also increased in response to acute EAA intake. All of the aforementioned skeletal muscle acylcarnitine basal concentrations were unchanged after 8 weeks of EAA supplementation. Prior to chronic supplementation, 3-hydroxy-isovaleryl-carnitine (3MC4OH), an intermediate of oxidative metabolism of leucine, was found to increase in response to acute EAA intake (Fig. 2, 70%, $p < 0.02$). Basal levels of 3MC4OH increased with supplementation.
(250%, p <0.05), however, there was no observable effect of acute EAA intake on skeletal muscle
3MC4OH concentrations following the 8-week supplementation period. It is notable that 3MC4OH
does not have a direct route to enter the TCA cycle, which may contribute to its accumulation in
muscle tissue (Fig. 3).

Succinyl-carnitine (C4DC) displayed a similar pattern of changes in response to EAA as 3MC4OH
(Fig. 4): increased C4DC concentration in response to the initial acute EAA intake (40%, p < 0.05),
increased basal concentrations of C4DC after 8 weeks of EAA supplementation (250%, p < 0.05), but
no increase in response to acute EAA intake after the 8-week supplementation period. C4DC is a
metabolic intermediate that is downstream of oxidative metabolism of a number of different amino
acids (Fig. 3). C4DC is also anaplerotic, acting to restore the TCA intermediate pool through a
succinyl-CoA to succinate pathway. Propionyl-carnitine (C3) is a precursor to C4DC and is also
considered anaplerotic. C3 was found to increase in response to acute EAA intake both before (135%,
p < 0.005) and after (143%, p < 0.01) the 8-week supplementation period (Fig. 4). Basal
concentrations of C3 had a modest increase (20%) in response to 8 weeks of EAA supplementation
that did not quite reach statistical significance (p < 0.06). Skeletal muscle concentrations of acetyl-
carnitine (C2) were not found to be increased either in response to acute EAA intake or to 8 weeks of
supplementation (Fig. 4).

*Medium- and long-chain acylcarnitines*

Skeletal muscle medium-chain (C4 through C10) and long-chain (C14 and C18) acylcarnitine
concentrations did not change significantly in response to acute EAA intake either before or after the
supplementation period (Fig. 5). Basal skeletal muscle medium-chain acylcarnitine concentrations
also did not show any changes in response to 8 weeks of EAA supplementation (Fig. 5). The basal
levels of myristoyl-carnitine (C14, 90% increase, p < 0.05) and stearoyl-carnitine (C18, 120% increase, p < 0.01) were found to increase in response to 8 weeks of EAA supplementation (Fig. 5).

**Organic Acids**

TCA intermediate concentrations in skeletal muscle were unchanged by acute EAA intake both before and after the 8-week EAA supplementation period (Supplemental Figure 1). Although the basal skeletal muscle concentrations of fumarate and malate increased by 44% (p < 0.01) and 115% (p < 0.002) (Fig. 6), respectively, in response to 8 weeks of EAA supplementation, there was no increase in the total sum of TCA intermediates (Supplemental Figure 2). A 32% increase in lactate in skeletal muscle (p < 0.04) was observed after the 8-week supplementation period. Muscle lactate and pyruvate concentrations were only found to increase in response to acute EAA intake after eight weeks of chronic EAA supplementation, not before (Fig. 7).

**Discussion**

Targeted metabolomic analysis of skeletal muscles biopsies obtained from hypertriglyceridemic older adults receiving EAAs both through acute intake and chronically for 8 weeks, revealed altered acylcarnitine and organic acid profiles. Acute EAA intake yielded increases in metabolic acylcarnitine intermediates of metabolism of EAA components, particularly BCAA catabolic intermediates, in skeletal muscle. The leucine metabolite most directly associated with 3MC4OH, beta-hydroxy-beta-methylbutyrate (HMB), has been implicated in the maintenance of muscle mass (20) and the increase of 3MC4OH may be related to a signaling pathway inhibiting muscle protein breakdown. The acute EAA intake mediated increase in 2MC3OH is notable for its structural relationship to 3-hydroxy-isobutyric acid, a metabolite of valine, which has been found to stimulate endothelial transport of FFA leading to lipid accumulation in skeletal muscle in mice (21).
Eight weeks of EAA supplementation resulted in increased basal concentrations of long-chain acylcarnitines. Previous *in vitro* studies found long-chain acylcarnitine accumulation in muscle tissue elicits an insulin resistant response (22). Additionally, 8-week EAA supplementation resulted in increased skeletal muscle concentrations of anaplerotic acylcarnitines C4DC and C3. Similar observations have been made in mice fed a high fat diet with BCAA (23) and are consistent with a hypothesis that increase BCAA catabolism leads to accumulation of fatty acid oxidation intermediates.

Although the total amount of TCA intermediates remained unchanged by 8 weeks of supplementation, a shift towards late stage TCA intermediates was observed. This distribution towards late-stage TCA intermediates is often reflective of anaplerosis (24, 25), and when considered alongside the increases in skeletal muscle anaplerotic acylcarnitines, this suggests that anaplerosis was increased following the 8-week EAA supplementation period. The increased skeletal muscle lactate concentrations after the 8-week period may arise from increased demand for NAD+ required for increased reliance on β-oxidation (26). Acute EAA intake after the 8-week supplementation period yielded increased skeletal muscle concentrations of lactate and pyruvate only after the 8-week supplementation period. This suggests there are alterations in energy metabolism arising from chronic EAA supplementation that do not present in the fasted state, but which become apparent when amino acids are ingested acutely.
Limitations

This investigation has several factors that may limit the conclusions drawn from it. First, there was no placebo control used, therefore placebo effects could be a possible explanation for our observations. However, the previously published controlled studies give us reason to believe that this is not a placebo effect (9, 10). Second, as a pilot study, the sample size was small (n = 6) thus the results are at increased risk of arising from type I error. Despite these limitations, we have a high degree of confidence in our results. First, the acute experiment was conducted both before and after the chronic period and the observed effects were confirmed in all but two of the thirty metabolites measured (Fig. 7) giving us a high degree of confidence that these results do not arise from type I error. The main findings that resulted from 8 week chronic supplementation were observed in two independent metabolites increasing our confidence that these did not arise from type I error: the increased long chain acylcarnitine concentrations was observed in both C14 and C18, and the increased late-stage TCA intermediates was observed in both FA and OA. The results described herein are statistically significant and fit within the larger context of our understanding of the physiological effects of EAA supplementation.

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Statement of authors’ contributions to manuscript. B.J.M., R.R.W. and E.B. designed research; B.J.M., N.M.H., E.C., I.Y.K, S.S. and G.A. conducted research; B.J.M. analyzed data; B.J.M and N.M.H. wrote paper; B.J.M. had primary responsibility for the final content. All authors read and approved the final manuscript.

Compliance with ethical Standards
Conflict of interest

The authors declare they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Figure/Table Captions

Figure 1: Overview of EAA supplementation experiment. An acute EAA-ingestion (3.5 hour) study was held before (week 0) and after (week 8) a chronic EAA supplementation period.

Figure 2: Box plots displaying skeletal muscle acylcarnitine (AC) concentrations before (white) and after (dark gray) acute EAA intake (n = 6) at week 0 and week 8 of daily EAA supplementation. AC displayed are intermediates of EAA oxidative metabolism. Labeling signifies paired t-test values of * = p < 0.05, ** = p < 0.01, and *** = p < 0.001.

Figure 3: Measured acylcarnitine species within catabolic pathways of amino acids present in EAA supplementation and anaplerotic routes into the TCA cycle through acetyl-CoA or succinyl-CoA.
Figure 4: Box plots displaying skeletal muscle acylcarnitine (AC) concentrations before (white) and after (dark gray) acute EAA intake (n = 6) at week 0 and week 8 of daily EAA supplementation. AC displayed are intermediates of EAA oxidative metabolism. Labeling signifies paired t-test values of # = p < 0.06, * = p < 0.05, and ** = p < 0.01.

Figure 5: Differential response to acute EAA intake before (Week 0) and after (Week 8) chronic EAA supplementation in skeletal muscle concentrations of medium and long chain ACs (n = 6). Labeling signifies paired t-test values of * = p < 0.05.

Figure 6: Effects of 8 weeks of EAA supplementation on basal skeletal muscle concentrations of TCA intermediates (in ng/mg). Late-stage TCA intermediates malate and fumarate were increased after 8 weeks of supplementation (dark gray) relative to initial levels (white) (n = 6). Labeling signifies paired t-test values of ** = p < 0.01.

Figure 7: Skeletal muscle LA and PA concentrations in response to acute EAA intake before (Week 0) and after (Week 8) chronic EAA supplementation (n = 6). Labeling signifies paired t-test values of * = P < 0.05, ** = p < 0.01, and *** = p < 0.001.
References


Skeletal Muscle AC Concentration (ng/mg)

**IsoButyryl Carnitine (2MC3)**
- Week 0: Basal
- Week 8: Basal

**3OH Butyryl Carnitine (C4OH)**
- Week 0: Basal
- Week 8: Basal

**2 Methyl Butyryl Carnitine (2MC4)**
- Week 0: Basal
- Week 8: Basal

**3OH IsoButyryl Carnitine (2MC3OH)**
- Week 0: Basal
- Week 8: Basal

**Isovaleryl Carnitine (3MC4)**
- Week 0: Basal
- Week 8: Basal

**3OH Isovaleryl Carnitine (3MC4OH)**
- Week 0: Basal
- Week 8: Basal

**Weeks of EAA Supplementation**
Skeletal Muscle AC Concentration (ng/mg)

**Acetyl Carnitine (C2)**

- Week 0: Basal 60 ng/mg, EAA Challenge 80 ng/mg
- Week 8: Basal 100 ng/mg, EAA Challenge 120 ng/mg

**Propionyl Carnitine (C3)**

- Week 0: Basal 2 ng/mg, EAA Challenge 3 ng/mg
- Week 8: Basal 3 ng/mg, EAA Challenge 4 ng/mg

**Succinyl Carnitine (C4DC)**

- Week 0: Basal 5 ng/mg, EAA Challenge 7 ng/mg
- Week 8: Basal 10 ng/mg, EAA Challenge 15 ng/mg

*Weeks of EAA Supplementation*

**References**

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Fumarate

Citrate

Succinate

Malate

Oxaloacetate

Isocitrate

TCA Cycle

α-Ketoglutarate

Succinyl-CoA

Succinate

Malate

Fumarate

Oxaloacetate

Citrate

α-Ketoglutarate

Succinyl-CoA

Succinate

Malate

Fumarate

Oxaloacetate

Citrate

α-Ketoglutarate

Succinyl-CoA

Succinate
Basal Acute EAAs

Skeletal Muscle Concentration (ng/mg)

Lactate

Pyruvate

Week 0 Week 8

Week 0 Week 8

*  **  ***
**Supplemental Table 1**: Summary of chromatographic and mass spectrometry parameters for identification and quantification of OBHA derivatized organic acids.

<table>
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<th>Analyte</th>
<th>Short Hand</th>
<th>Retention Time (min)</th>
<th>MRM Transition</th>
<th>Internal Standard</th>
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<tr>
<td>α-ketoglutarate</td>
<td>αKG</td>
<td>5.26</td>
<td>462/339</td>
<td>αKG (D₆)</td>
</tr>
</tbody>
</table>
**Supplemental Table 2:** Summary of chromatographic and mass spectrometry parameters for identification and quantification of OBHA derivatized organic acids.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Short-hand</th>
<th>Retention Time (min)</th>
<th>MRM Transition</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-L-Carnitine</td>
<td>C2</td>
<td>1.2</td>
<td>204/85</td>
<td>C2 (D₃)</td>
</tr>
<tr>
<td>Succinyl-L-Carnitine</td>
<td>C4-DC</td>
<td>1.4</td>
<td>292/85</td>
<td>C2 (D₃)</td>
</tr>
<tr>
<td>3 Hydroxy Isovaleryl-L-Carnitine</td>
<td>3M-C4-OH</td>
<td>1.6</td>
<td>262/85</td>
<td>C2 (D₃)</td>
</tr>
<tr>
<td>3 Hydroxy Butyryl-L-Carnitine</td>
<td>C4-OH</td>
<td>1.35</td>
<td>248/85</td>
<td>C2 (D₃)</td>
</tr>
<tr>
<td>3-Hydroxy Isobutyryl-L-Carnitine</td>
<td>2M-C3-OH</td>
<td>1</td>
<td>248/85</td>
<td>C2 (D₃)</td>
</tr>
<tr>
<td>Glutaryl-L-Carnitine</td>
<td>C5-DC</td>
<td>1.5</td>
<td>276/85</td>
<td>C3 (D₃)</td>
</tr>
<tr>
<td>Propionyl-L-Carnitine</td>
<td>C3</td>
<td>1.8</td>
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<td>C3 (D₃)</td>
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<tr>
<td>Isobutyryl-L-Carnitine</td>
<td>2M-C3</td>
<td>2.5</td>
<td>232/85</td>
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</tr>
<tr>
<td>Butyryl-L-Carnitine</td>
<td>C4</td>
<td>2.7</td>
<td>232/85</td>
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</tr>
<tr>
<td>2MTBL</td>
<td>2M-C4</td>
<td>3.45</td>
<td>246/85</td>
<td>3M-C4 (D₉)</td>
</tr>
<tr>
<td>Isovaleryl-L-Carnitine</td>
<td>3M-C4</td>
<td>3.6</td>
<td>246/85</td>
<td>3M-C4 (D₉)</td>
</tr>
<tr>
<td>Hexanoyl-L-Carnitine</td>
<td>C6</td>
<td>5.71</td>
<td>260/85</td>
<td>C6 (D₃)</td>
</tr>
<tr>
<td>Octanoyl-L-Carnitine</td>
<td>C8</td>
<td>6.25</td>
<td>288/85</td>
<td>C8 (D₃)</td>
</tr>
<tr>
<td>Decanonyl-L-Carnitine</td>
<td>C10</td>
<td>6.75</td>
<td>317/85</td>
<td>C10 (D₃)</td>
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<tr>
<td>Lauroyl-L-Carnitine</td>
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<td>7.4</td>
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<td>Myristoyl-L-Carnitine</td>
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<td>Stearoyl-L-Carnitine</td>
<td>C18</td>
<td>8.3</td>
<td>429/85</td>
<td>C18(D₃)</td>
</tr>
</tbody>
</table>
Supplemental Figure 1: Effects of 3.5 h of EAA acute intake on skeletal muscle concentrations of TCA intermediates (in ng/mg; n=6).
Supplemental Figure 2: (Left) Effects of 3.5h acute EAA intake on sum of skeletal muscle concentrations of TCA intermediates (in ng/mg; n=6). (Right) Effects of 8 week EAA supplementation on sum of skeletal muscle concentrations of TCA intermediates (in ng/mg; n=6).