The role of adiposity in the association of branched-chain amino acids and metabolic and clinical markers in healthy adults on controlled diets.

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Abstract

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Branched-chain amino acids (BCAAs) leucine, isoleucine, and valine have been positively associated with adiposity and insulin resistance. Targeted metabolomic data from plasma samples of healthy adults (n=40 men, n=40 women) aged 18-45 years old derived from a randomized crossover feeding trial (Carbohydrates and Related Biomarkers-ClinicalTrials.gov Identifier: NCT00622661) were used to further elucidate why BCAAs are elevated in settings of increased adiposity and insulin resistance. Twelve metabolites were selected from a combination of a literature search and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway review for cross-sectional analysis. Linear mixed models were used to evaluate associations between (1) adiposity and metabolites related to BCAA catabolism, (2) adiposity, HOMA-IR, and CRP, and (3) markers of BCAA catabolism, adipokines (leptin and adiponectin), and markers of inflammation (CRP, IL-6, and SAA). Adiposity was positively associated with leucine (p=0.043), isoleucine (p=0.035), valine (p=0.034), pyruvate (p=0.015), alanine (p=6e-4),

glutamic acid (p=8e-7), and isovaleric acid (p= 2e-4). Conversely, malonic acid concentration was found to be inversely associated with body adiposity (p=0.014). These results support the hypothesis that elevated BCAAs are part of a metabolic signature of obesity and reductions in BCAA catabolism at the first two steps of BCAA catabolism likely drive elevations in plasma BCAA concentrations. Serum glucose, insulin, HOMA-IR score, and CRP were all positively associated with adiposity (p=2.3e-3, p=7.88e-6, p=3.65e-6, p=5e-3, respectively). These results were significant even after adjustments for plasma leucine, isoleucine, and valine concentration, and carbohydrate intake. Finally, plasma leptin was positively associated with leucine (p=0.042), isoleucine (p=0.045), valine (p=0.021), and glutamic acid (p= 0.027) and these associations could be related to insulin resistance and regulation of plasma insulin levels. Leptin was inversely associated with malonic acid which may be related to the opposing roles leptin and malonic acid-derived malonyl-CoA play in fatty acid metabolism. These exploratory metabolomic results provide key connections between metabolites related to BCAA catabolism, adiposity, inflammation, and adipokine activity. These results have broad applications towards understanding the complex signaling effects of BCAAs on the human metabolome.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE: LITERATURE REVIEW

An Overview of BCAAs

Branched-chain amino acids (BCAAs), leucine, isoleucine, and valine, are essential amino acids derived from the diet that are important for many physiological processes in the human body. These hydrophobic amino acids are crucial in globular protein (i.e., hemoglobin, immunoglobulins, insulin) formation and stability,[1] muscle protein synthesis,[2] and act as signaling molecules for a range of metabolic processes.[3] Inborn errors of BCAA metabolism can result in the development of maple syrup urine disease, and have also been linked to autism spectrum disorder with epilepsy.[4] Plasma BCAA concentrations are altered in several human disease states. Conditions linked to protein-calorie malnutrition and elevated protein catabolism like liver diseases, sepsis, trauma and burns typically result in low plasma BCAA concentrations. BCAA supplementation has been examined as a possible treatment method for these conditions.[5] Conversely, conditions linked to obesity and insulin resistance like cardiovascular disease and T2DM result in elevated plasma BCAA concentrations.[5]

Dietary intake of protein is the main way of acquiring BCAAs. All protein-containing foods have BCAAs, and animal proteins and dairy products are the richest sources.[6] Plant-based sources of BCAAs include legumes, nuts, and pulses, and some starchy vegetables.[6] BCAAs are also ingested as supplements, particularly among bodybuilders and strength athletes.[7] Upon ingestion of a protein-containing meal, roughly 50% of the BCAAs in that meal enter systemic circulation from the splanchnic capillary bed.[8] Circulating levels of BCAAs increase after a protein-rich meal and decline to baseline (approximately 200 of μM valine, 100 μM of leucine, and 60 μM of isoleucine) within three hours,[8] indicating homeostatic regulation of plasma

BCAA levels.[9] Circulatory BCAAs are up taken by various tissues and undergo deamination and oxidation to form various metabolites and proteins.[10]

Key steps of BCAA Catabolism

Several steps of BCAA catabolism result in metabolic intermediates that can serve as plasma indicators of BCAA degradation. Unlike many other amino acids which are metabolized in the liver, BCAA catabolism typically occurs in extrahepatic tissue, particularly muscle. [11] This is likely due to low hepatic transamination activity of branched chain aminotransferases (BCAT), the first enzyme required for leucine, isoleucine, and valine catabolism.[12] This results in rapid systemic elevation of circulating BCAAs after ingestion and absorption in the epithelial cells of the small intestine. Skeletal muscle is the most frequent site of BCAA catabolism due the high energy demand of this tissue. High concentrations of the BCAT enzyme exist in skeletal muscle in order to utilize BCAAs for energy production.[13] Many other tissues are capable of BCAA catabolism including the brain, adipose tissue, liver and kidneys.[1] In the mitochondrial matrix, BCAT facilitates transamination of BCAAs to form branched chain alpha keto acids.[14] This reversible reaction requires a nitrogen acceptor alpha-ketoglutarate (aKG) which undergoes further transamination to produce glutamate, a byproduct of BCAA catabolism.[14] Glutamate then acts as an amino group source to form non-essential amino acid alanine from pyruvate.[11] Pyruvate is the product of glycolysis and the driver of ATP production via oxidative phosphorylation.[15] Glutamate can also generate glutamine via activity of glutamine synthetase, an enzyme that supports ammonia detoxification.[16]

Branched chain a-keto acids a-ketoisocaproate, a-keto-b-methylvalerate, and a-ketoisovalerate undergo oxidative decarboxylation in the branched chain alpha-keto acid amino dehydrogenase complex (BCKDH) to covalently add a coenzyme-A (CoA) group to the oxidized

branched chain a-keto acid.[17] All subsequent BCAA catabolic intermediates with a CoA group are trapped in the mitochondria due to the bulky, hydrophilic nature of CoA with the exception of 3-hydroxyisobutyrate, an intermediate of valine degradation.

After BCKDH decarboxylation, each BCAA follows a unique catabolic pathway that resembles fatty acid oxidation. Each carbon of BCAA is lost as CO_2 or the amino acid enters the tricarboxylic acid (TCA) cycle as succinyl-CoA (valine, isoleucine) or acetyl-CoA (leucine).[10] Propionyl-CoA, a derivative of propionate and intermediate of isoleucine and valine catabolism, has been shown to contribute to odd chain fatty acid synthesis [18,19]. Propionyl-CoA can also be converted to methylmalonyl-CoA, a derivative of methylmalonate, and subsequently succinyl-CoA for use in the TCA cycle.[20] Propionyl-CoA is also converted to C3-acylcarnitine, a byproduct of isoleucine and valine metabolism. Another acylcarnitine, C5-acylcarnitine, is formed as a byproduct of leucine and isoleucine catabolism.[1] Both C3 and C5 acylcarnitines have been elevated in participants with insulin resistance and obesity.[21] Acylcarnitines are produced from long-chain acyl-CoAs via activity of carnitine palmitoyltransferase-1 (CPT1).

BCAA catabolic intermediates may offer unique perspectives on the BCAA catabolic flux. 3-hydroxyisobutyrate (3-HIB) and acetoacetate are commonly researched BCAA catabolic intermediates of valine and leucine degradation, respectively.[10] Neither of these compounds are bound to a CoA group, so they can be released from the mitochondria and are detectable in plasma.[22,23] Other BCAA catabolites are less researched. Prior to BCKDH activity, branched chain a-keto acids can be converted to α-hydroxy keto acids which are detectable in urine.[24] After BCKDH oxidation many catabolites bound to CoA can be released from the mitochondria as 3-hydroxy acids, including 3-hydroxyisovaleric acid which can be detectable in plasma.

Obesity, diet, and reductions in BCAA catabolism contribute to elevated plasma BCAA concentrations.

Elevated plasma BCAA and acylcarnitine concentrations have been observed in obese individuals and those consuming a BCAA-rich diets.[6] Impairments in BCAA catabolism in adipose tissue and skeletal muscle in the context of obesity and insulin resistance may contribute to these observations. Metabolite profiling of the offspring of Framingham Study participants indicated that plasma BCAA levels served as a risk factor for developing T2DM even before weight gain.[25] Several studies and review articles [3,21,26–29] have observed similar trends. Furthermore, comparisons of metabolomic profiles between lean, overweight, and obese humans show elevated BCAAs are associated with increased adiposity.[30] Short chain acyl-carnitines (C3 and C5 acylcarnitine) produced by BCAA catabolism have been associated with obesity and T2DM.[21,31] Acylcarnitine buildup in the cell has been associated with elevated plasma BCAAs, and are thought to disrupt fatty acid oxidation in mitochondria, reduce insulin sensitivity in skeletal muscle, and subsequently lead to the development of insulin resistance.[32]

Diet, sex, and supplementation have also been shown to impact plasma BCAA concentrations.[33,34] Wang and colleagues observed lower circulating levels of plasma BCAAs in those consuming vegetarian diets, typically lower in protein, than those consuming omnivorous diets.[34] Additionally, a cross-sectional analysis conducted by Merz and colleagues of participants from the Karlsruhe Metabolomics and Nutrition (KarMeN) study showed that diets with high consumption of animal-based proteins and low consumption of plant-based proteins were associated with significantly elevated plasma BCAA concentrations.[33] Dietary patterns high in animal protein likely result in consistent introductions of BCAAs into circulation.[35] Habitual plasma exposure to BCAAs may reduce the homeostatic regulatory capacity of the body to

maintain specific concentrations of BCAAs in the plasma after a protein-rich meal. Sex also impacts plasma BCAA concentrations, with men typically having greater plasma BCAA concentrations than women. Plasma BCAA concentrations have been associated with muscle protein degradation, and males typically have greater muscle mass than females.[35] It is unclear how being intersex or receiving gender affirmation surgeries impacts plasma BCAA concentrations. BCAA supplementation is popular amongst strength-training athletes due to claims that their ingestion encourages muscle protein synthesis and reduces post-exercise muscle fatigue and soreness.[7] A meta-analysis conducted by Fedewa and colleagues identified 8 studies that indicated BCAA supplementation significantly reduced delayed onset muscle soreness after exercise compared to no BCAA supplementation.[36]

In the setting of obesity and insulin resistance, disruptions in several points of BCAA catabolism in adipose tissue and skeletal muscle may contribute to elevations in plasma BCAAs. BCAT and BCKDH activity comprise steps one and two of BCAA catabolism with BCAT activity being irreversible and BCKDH activity being reversible.[37] This second step of BCAA catabolism is regulated by the phosphorylation and dephosphorylation of BCKDH kinase. BCKDH is also allosterically suppressed by branched chain a-keto acids, particularly a-ketoisocaproic acid (a-KIC).[17,37] She et al. demonstrated significant increases in (BCAT) and BCKDH activity in subcutaneous and visceral adipose tissue post Roux-en-Y gastric bypass surgery.[38] Participants experienced an average fat loss of 56 kilograms over the course of 17 months post-surgery. Investigators were also able to show that in models of obesity, adipose tissue seems to be the main contributor to alterations in BCAT and BCKDH activity.[38] Additionally, Herman and colleagues observed that transplantation of normal adipose tissue into mice who were globally unable to metabolize BCAAs resulted in significant reductions in

circulating BCAAs in both fasted and fed states.[39] These studies indicate that obesity likely results in reduced activity of BCAT and BCKDH within adipose tissue, resulting in increased BCAAs in plasma circulation.

Impairments in BCAT and BCKDH activity have also been observed in the skeletal muscle of humans and rats. In humans with T2DM and subsequent insulin resistance, genes related to BCAT and BCKDH activity in skeletal muscle were significantly downregulated compared to participants with normal glucose tolerance.[40] Additionally, Lerin and colleagues conducted metabolomic analysis on skeletal muscle tissue of insulin resistant participants and observed significant decreases in branched chain a-keto acids ketoisovaleric acid and ketoisocaproate compared to insulin sensitive muscle samples. Furthermore, David and colleagues observed impairments in transamination and oxidative deamination of BCAAs in the skeletal muscle of insulin-resistant, non-obese rats.[41] These studies indicate that both adipose tissue and skeletal muscle contribute to disruptions in BCAA catabolism in the context of obesity and insulin resistance.

Proposed mechanisms for insulin resistance development in response to elevated plasma BCAAs

BCAAs have been linked to several different pathways of insulin resistance development. Several review articles [28,29,42–45] have highlighted the central roles of mammalian target of rapamycin complex 1 (mTORC1)/p70s6 kinase (S61k) and insulin receptor substrate-1 serine/tyrosine (IRS1 Ser/Tyr) in these mechanisms. A clinical trial conducted by Tremblay and colleagues used skeletal muscle biopsies of healthy males taken before and after postprandial peripheral insulinemia induced by a somatostatin insulin-glucose clamp. Compared to saline infusions, infusion of amino acids including BCAAs significantly increased the activity of S6K1 and subsequent serine phosphorylation of IRS1 (Ser312).[46] These findings are further supported

by various mice experiments which demonstrated elevated mTORC1 phosphorylation when plasma BCAAs were consumed.[17,21,47,48] Phosphorylation of mTORC1 led to serine phosphorylation of S6k1 and subsequently, IRS1 which reduced the number of insulin-responsive receptors, predominantly in skeletal muscle.[17,21,47,48] Newgard et al were also able to demonstrate that BCAA-mediated S6K1 activation and IRS1 degradation can occur independently of increased adiposity as evidenced by a linear relationship between BCAA metabolites and homeostatic model assessment of insulin resistance (HOMA-IR) data adjusted for obesity, age, race, and sex.[21] This ultimately resulted in the development of insulin resistance derived from amino acids.

Effects of BCAAs on adipokines and markers of inflammation

BCAAs are thought to have regulatory effects on the release of adipokines leptin [49,50] and adiponectin. Lynch and colleagues demonstrated in rats that dietary leucine is the main nutrient contributor to postprandial rises in leptin.[49] Leptin is a peptide satiety hormone released from adipocytes in response to food intake. Leptin acts on the hypothalamus to regulate anorexigenic and orexigenic neuropeptides proopiomelanocortin and neuropeptide-Y by upregulating proopiomelanocortin signaling and suppressing neuropeptide-Y signaling resulting in satiety.[51] This adipokine is most known for its regulation of satiety, energy expenditure and body weight. Other regulatory effects of leptin include insulin secretion from pancreatic beta islet cells [51] and regulating bone mass through hormones in the hypothalamus-pituitary-adrenal axis.[52]

Adiponectin is predominantly released from adipocytes and leads to increased insulin sensitivity by promoting beta cell survival in the pancreas and reduced glucose output in the liver.[53] Reduced concentrations of adiponectin have been associated with metabolic

dysregulation [53], and adiponectin introduction has been shown to correct dysregulated BCAA catabolism resulting from the consumption of a high-fat diet in mouse models.[54]

C -reactive protein (CRP), interleukin-6 (IL-6), and serum amyloid-A (SAA) are all markers of inflammation in a variety of tissues. CRP is an acute phase inflammation protein synthesized primarily in the liver and is used as a clinical marker for inflammation.[55] There are few studies that directly compare plasma BCAA levels to plasma CRP, however the proposed BCAA metabolic regulatory pathway is tied to CRP through the activity of mTORC1 and IL-6.[47] IL-6 is a pro-inflammatory cytokine produced in response to acute stressors like infections, and tissue damage, and chronic stressors like cardiovascular disease and diabetes.[56] Notably, adipose tissue contributes to nearly one-third of circulating IL-6.[57] In the liver, IL-6 impairs early insulin receptor signal transduction and downstream insulin action in hepatocytes. Furthermore, effects of IL-6 seem to depend on acute versus chronic presentations of this cytokine, with chronic circulation of IL-6 being associated with insulin resistance.[57] BCAA signaling activity is mediated via mTORC1, which is an upstream signaling kinase for IL-6.[43,47] IL-6 is the main mediator of hepatic CRP production [58], making it likely that plasma BCAAs have an indirect impact on serum IL-6 and CRP levels. SAA proteins are a group of apolipoproteins spanning multiple closely related genes that are integral to acute phase inflammatory responses.[59] Like CRP, the literature correlating plasma BCAA levels and SAA responses is sparse. However, IL-6 is also a mediator of SAA production [60], contributing to the idea that BCAA levels may indirectly impact SAA synthesis. A standing hypothesis proposed by Newgard et al. postulates that elevated BCAAs coupled with elevated adipocytokines, metabolic, and inflammatory markers leptin, adiponectin, fasting glucose, plasma insulin, CRP, IL-6, and SAA are part of a metabolic signature of adiposity and insulin resistance.[21]

Metabolomics in nutrition studies

Metabolomics is defined as the large-scale study of small molecules found in living organisms, commonly referred to as metabolites.[61] The most common techniques used are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Preliminary mass separation steps gas chromatography or liquid chromatography are typically combined with mass spectrometry.[62] Study designs frequently utilize group comparison where the metabolic profile of one group is compared to the metabolic profile of another.[63] In either form of metabolomic assessment, metabolites are quantified in relative abundances. This means that the observed amount of each metabolite analyzed is the abundance of a particular metabolite relative to the abundances of every other metabolite measured in the sample, rather than the absolute concentration of a metabolite.[63] Relative abundances allow for conclusions to be drawn about metabolite differences within a study population, but limit the comparability of these abundances to studies that measure absolute concentrations of metabolites of interest. Despite this drawback, high-throughput technologies like metabolomics analysis have made it possible to assess the associations of an exposure on thousands of metabolites simultaneously. Additionally, this form of analysis addresses the complex impact dietary patterns elicit on the human metabolome and allow for the identification of new biomarkers that are reflective of human health and disease.[62]

CHAPTER TWO: INTRODUCTION

The role of adiposity and branched chain amino acids (BCAAs) leucine, isoleucine, and valine in the development of insulin resistance has been widely studied.[3,21,25,28,29,64–68] Interestingly, elevated plasma BCAAs have been consistently observed in humans with type II diabetes (T2DM) [25,69–71], a condition characterized by the development of insulin resistance. A combination of prolonged consumption of BCAA rich diets [33,34] and disruptions in BCAA catabolism [18,19,29,38,64,66,72–74] likely contribute to their elevation in plasma. Some studies postulate that elevated BCAAs may increase risk of developing insulin resistance prior to increases in body fat percentage [9,46,47,75–78] while others hypothesize elevated BCAAs occur as a byproduct of obesity.[21,30,64,66,79–81] A variety of mechanisms have been proposed to explain these observations [18,32,46,47,64,78,82] many of which are related to the proposed signaling effects of BCAAs and subsequent metabolites of BCAA catabolism on adipokines, kinases, and cytokines involved in processes of insulin resistance, inflammation, and adipokine activity.[3,25,27–29,38,49,83]

Although studies have explored associations between some BCAA catabolic intermediates and markers of metabolism and inflammation, few connect the proposed activity of these intermediates with insulin resistance. Furthermore, none of these studies have examined associations between BCAA catabolites, adipokines, metabolic and inflammatory markers in healthy adults in the context of controlled diets, minimizing confounding by dietary intake.

The Carbohydrates and Related Biomarkers (CARB) study conducted between June 2006 and July 2009 at the Fred Hutchinson Cancer Research Center (Fred Hutch) (ClinicalTrials.gov Identifier: NCT00622661) was a randomized controlled crossover feeding trial in healthy adults that assessed the effects of dietary glycemic load on markers of chronic disease susceptibility, systemic inflammation, metabolism, and incretin concentrations. Several articles were published

using data from this clinical trial [84–87], including a targeted metabolomic analysis conducted by Navarro and colleagues which evaluated the effects of these dietary patterns on the targeted plasma metabolome.[84] In a post-hoc analysis of Navarro's study, associations were observed between plasma isoleucine and valine and HOMA-IR score amongst study participants independent of adiposity. This observation led to the hypothesis that even in healthy adults with no identifiable metabolic dysregulation, elevated plasma BCAAs may predict insulin resistance independent of body fat percentage.

The purpose of the present analysis is to utilize targeted metabolomic data from the CARB study[84] to evaluate the cross-sectional associations between metabolites related to BCAA catabolism, and adiposity, with markers of insulin resistance, adipocytokines, and inflammation in healthy adults on controlled diets. This analysis was structured around three converging aims. In aim one, adiposity was compared with metabolites related to BCAA catabolism in order to further elucidate this relationship. In aim two, adiposity was compared with markers of metabolism adjusting for plasma BCAA in order to broadly evaluate the impact of adiposity, irrespective of BCAA abundances, on metabolism in healthy adults. In our final aim, plasma concentrations of adipokines and markers of inflammation were compared with metabolites related to BCAA catabolism in order to explore how BCAAs and their catabolic intermediates may impact the regulation and function of adipokines and cytokines in healthy adults on controlled diets. Results from each aim were evaluated in the context of insulin resistance to further elucidate the complex role BCAAs play in insulin resistance development.

CHAPTER 3: METHODOLOGY

Study Design

Participant and biological data for this analysis were drawn from The Carbohydrates and Related Biomarkers (CARB) study conducted between June 2006 and July 2009 at the Fred Hutchinson Cancer Research Center (Fred Hutch) (ClinicalTrials.gov Identifier: NCT00622661). This randomized controlled crossover feeding trial assessed the effects of dietary glycemic load on markers of chronic disease susceptibility, systemic inflammation, insulin resistance, and adipokine concentrations. Participants received two eucaloric experimental diets of low glycemic load and high glycemic load in random order. Each diet was consumed for 28 days with a 28-day washout period in between diets. All foods for each diet period were prepared by the Human Nutrition Laboratory at Fred Hutch in a standardized manner using strict hazard analysis critical control point procedures. During the washout period, participants consumed their own food. Participants were instructed to consume only food and beverages provided in this study during the study period except for plain tea and coffee. Participants ate dinner daily at the Human Nutrition Laboratory from Monday through Friday. Breakfast, lunch, snacks, and weekend meals were eaten at home. Participants kept a daily food log of all provided and non-provided foods consumed. Any unconsumed food was returned, weighed, and recorded at Fred Hutch. Additional details on study diet have been discussed in previous publications using CARB study data[84,86,87]

CARB Study Participants

Healthy, non-smoking men (n=40) and women (n=40) aged 18-45 in Seattle and surrounding areas were recruited through the following mediums: advertisements in local newspapers, the Fred Hutch website, college newsletters, flyers distributed around local

college/university campuses, and through local and student organizations working with African American and Hispanic communities.

Inclusion Criteria

Participants had to be healthy males and female subjects between the ages of 18 to 45 with a BMI between 18.5 kg/m^2 to 24. 9 kg/m^2 (normal weight) and between 28.0 kg/m^2 to 39.9 kg/m^2 (overweight). Participants were willing to refrain from alcohol during the study and able to come to the Fred Hutch in Seattle every weekday night for dinner.

Exclusion Criteria

Those who were: (1) younger than 18 years of age or older than 45 years of age, (2) did not fit into one of the study weight groups, (3) had diseases that were treated by diet and/or medications including but not limited to diabetes, (4) kidney disease, heart disease, (5) taking prescription medications every day (this includes women taking birth-control pills, shots, patch or IUD with hormones), (6) diagnosed with or treated for cancer within the previous five years (except those with a diagnosis and/or treatment of non-melanomatous skin cancer are eligible), (7) pregnant or breastfeeding or planning a pregnancy in the next 3 months, (8) using any tobacco products on a daily basis (cigarettes, pipes, cigars, chewing tobacco), (9) using recreational drugs, (10) drinking the following amount of alcohol almost every day: two or more cans/bottles of beer OR two or more glasses of wine OR three or more ounces of hard liquor, (11) unable (e.g., food allergy or intolerances) or unwilling to consume the foods that are part of the feeding study diet were excluded from the CARB study.

Data Collection

Anthropometrics and Body Composition

Baseline height was taken to the nearest 0.5 cm using a wall-mounted stadiometer. Baseline weight and weights three times a week during each diet period were taken to the nearest 0.5 kg using a calibrated digital scale. Body composition (% body fat and % lean mass) were determined using whole body DXA scanning.[88]

Blood Collection

Blood samples were collected from each participant at baseline and on day 1 and 28 of each diet period. All samples were collected after a minimum 12-hour overnight fast, processed, and stored at -80°C using a standard protocol. Plasma preparation used for metabolomics assays, and serum preparation for HOMA-IR, adiponectin, and inflammation markers have been outlined previously. [84,87]

Metabolomics profiling of Plasma Samples

Metabolomic data in this analysis were analyzed from metabolomic profiles collected by Dr. Navarro at the Fred Hutch assessing the impact of low and high glycemic load diets on a variety of metabolites and nutrients. In her analysis targeted metabolomics were carried out using liquid chromatography-tandem mass spectrometry (LC-MS/MS) platform at the University of Washington's Northwest Metabolomic Research Center. Please refer to Dr. Navarro's study for a complete LC-MS/MS protocol.[84]

Metabolite Selection for Present Analysis

A total of 121 metabolites were reliably quantified using targeted metabolomic techniques. A PubMed literature search using the following search terms was used to identify potential metabolites associated with BCAA catabolism:

"branched chain amino acid catabolism", "branched chain amino acids AND insulin resistance", "branched chain amino acids AND adiposity", "branched chain amino acids AND adipokines", "branched chain amino acids AND diabetes", "branched chain amino acids AND inflammation", "branched chain amino acids AND diet", and "branched chain amino acids AND incretins".

Articles written in English were included in this literary review. The genes for relevant metabolites and proteins of interest from the literary review were entered into KEGG to identify relevant pathways. Based on relevant literature and KEGG pathway information, the following 12 metabolites related to BCAA catabolism were selected from Dr. Navarro's metabolomic dataset [84]: Propionate, Succinate, Malonic Acid, Acetyl Carnitine, Isovaleric Acid, Hydroxy isovaleric Acid, Methylmalonate, Glutamic Acid, Alanine, Ketoglutaric Acid, Pyruvate, and Inositol. A few metabolites of interest related to BCAA degradation were identified during this literature review but were not included in this analysis due to lack of metabolic profile data. These metabolites included acetoacetate, 3-hydroxyisobutyrate, and C3/C5 acylcarnitine.

Insulin Resistance, Inflammation, and Adiposity Biomarker concentrations

Serum samples were analyzed for concentrations of high-sensitivity C-reactive protein (*hs*-CRP), adiponectin, glucose, insulin, IL-6, SAA, and leptin at baseline, and on days 1 and 28 of each diet. Latex-enhanced nephelometry high sensitivity analysis conducted using the Nephelometer II analyzer at the University of Washington Medical Center to assess CRP and SAA concentrations. ELISA was used to assess IL-6, leptin, and adiponectin concentrations. Roche reagents were used to enzymatically determine serum glucose levels on a Roche Module P Chemistry autoanalyzer, and serum insulin levels were assessed at the Diabetes Endocrinology Research Center Immunoassay Core Laboratory at the University of Washington. Homeostatic

Model Assessment for Insulin Resistance (HOMA-IR), a measure of insulin resistance, was calculated by taking the product of fasting glucose and insulin in mg/dL and dividing this value by 405. [89]

Statistical Analysis

The goals of this analysis were to cross-sectionally test for associations between body fat percentage, metabolites related to BCAA catabolism, markers of metabolism, adipokines, and markers of inflammation. Of the 80 participants, two had missing body fat percentage and BCAA catabolite data, leaving a total of 78 participants for these analyses. Each statistical aim was as follows:

Aim 1: To evaluate associations between adiposity and concentrations of key plasma metabolites linked to BCAA catabolism in healthy adults on controlled diets.

Aim 2: To evaluate associations between adiposity and plasma markers of inflammation and insulin resistance (or HOMA-IR) in healthy adults on controlled diets.

Aim 3: To evaluate associations between BCAA catabolites, adipocytokines, and markers of inflammation in healthy adults on controlled diets.

Histograms and Shapiro-Wilks tests were used to evaluate the distribution of each variable of interest. Body fat percentage was normally distributed in this dataset and treated as a continuous variable throughout each set of analyses. Other continuous exposure and outcome variables underwent natural log transformation to reduce left skew.

In Aim 1, body fat percentage served as the exposure variable while metabolites related to BCAA catabolism (Table 1) served as outcome variables. All outcome variables were left skewed, so natural log transformation was performed to generate a distribution closer to normal.

Notable adjustments include leucine, isoleucine, valine, and alanine intake. See Table 1 for all covariates in this analysis.

In Aim 2, five linear mixed models were run with body fat percentage as the exposure variable and markers of insulin resistance and inflammation including serum glucose, insulin, *hs*-CRP, adiponectin, IL-6, SAA, and leptin as outcome variables (Table 1). These outcome variables were skewed, so natural log transformation was performed. Additionally, *hs*-CRP observations that were below the limit of detection were reported as nonnumerical values (<0.2). These data points were replaced by half of the data column minimum. Results were notably adjusted for plasma leucine, isoleucine, and valine abundance as well as carbohydrate intake. See Table 1 for a full list of covariates in this analysis.

In Aim 3 (Table 1), a total of 60 linear mixed models were run. Each exposure variable was tested against metabolites related to BCAA catabolism, resulting in 12 different looped linear mixed models for each of the five exposure variables. Both exposure and outcome variables were natural log transformed for skew. Data columns for *hs*-CRP, IL-6, and SAA contained non-numerical values which were replaced with half of the column minimum. While we recognize that many tests were run, due to the exploratory nature of this analysis, an adjustment for multiple comparisons was not made. See Table 1 for a full list of all covariates in this analysis.

Linear mixed models for each aim were carried out using R Studio base package [90] and the following additional packages: lme4 [91], lmerTest [92], pylr [93] , and limma.[94] For each aim, results were presented using beta coefficients and standard error with p-values. Significance was set at 0.05, two-sided.

Table 1: Outline of Statistical Analysis Plan

	Exposure	Outcome	Covariates
Specific Aim One Body fat Percentage and BCAA catabolites	Body Fat Percentage (continuous)	Leucine, Isoleucine, Valine, Propionate, Succinate, Malonic Acid, Acetyl-Carnitine, Isovaleric Acid, Hydroxy isovaleric Acid, Methylmalonate, Glutamic Acid, Alanine, Ketoglutaric Acid, Pyruvate, and Inositol	Study diet, diet sequence, sex, age, sample batch, leucine, isoleucine, valine, and alanine intake.
Specific Aim Two Body fat percentage and markers of insulin resistance	Body Fat Percentage (continuous)	Plasma glucose, plasma insulin, and HOMA-IR score	Study diet, diet sequence, sex, age, sample batch, plasma leucine, isoleucine, and valine abundance, average consumed carbohydrates.
Specific Aim Three BCAA catabolites and incretins, adipocytokines, and markers of inflammation, and	Leucine, Isoleucine, Valine, Propionate, Succinate, Malonic Acid, Acetyl-Carnitine, Isovaleric Acid, Hydroxyisovaleric Acid, Methylmalonate, Glutamic Acid, Alanine, Ketoglutaric Acid, Pyruvate, and Inositol	Adipocytokines: Leptin and Adiponectin Inflammation Markers: *hs*-CRP, IL-6, and SAA	Study diet, diet sequence, sex, age, sample batch, and body fat percentage

CHAPTER 4: RESULTS

Participant characteristics for the 78 CARB participants are included in Table 2. Mean participant age was 29.6±8.14 years with a range of 18-45. 51% of participants were female and 49% were male. Nearly half of participants were non-Hispanic White, 21% were Hispanic, and 17% were African American. Mean body fat percentage was 32.8 (±11.8%) (Figure 2), and mean weight was 81.1 (±21.6) kg (Figure 3).

Participant intake data can be found in Table 3. Each diet was comparable in kcals, carbohydrates, total protein, and BCAA consumption. Fiber intake differed most between diets, with the low glycemic load diet containing more soluble and insoluble fiber than the high glycemic load diet. Compared to NHANES 2017-2018 dietary intake data [95,96], each participant consumed more calories, carbohydrates, protein, and total fiber than the average American (Table 3).

Table 2. Participant Demographic Information

Demographic Characteristic	**All Participants, *n=80***
Age	30±8 (range 18-45)
Sex	
Male	50%
Female	50%
Race/Ethnicity	
Non- Hispanic White	49%
Hispanic	21%
African American	17%
Asian/Pacific Islander/Native American	13%
% Body Fat	33±12 (range: 6-57%)
Weight, kg	81±22

Table 3. Average selected dietary components for participant intake within the CARB study.

Intake Category	**Low glycemic load diet ±SD**	**High glycemic load diet ±SD**	**Average of Both Diets**	**Average Intake Amongst Americans 20 and over (NHANES 2017-2018)**
Energy (kcal/d)	2574±479	2585±449	2579	2155
Carbohydrate (g/d)	388±71	369±63	378	248
Fat (g/d)	88±17	86±16	87	88
Protein (g/d)	98±19	97±17	98	82
Leucine (g/d)	7±1.3	7±1.3	7	-
Isoleucine (g/day)	4±0.8	4±0.8	4	-
Valine (g/day)	5±0.9	5±0.9	5	-
Alanine (g/day)	4±0.8	5±0.8	4	-
Estimated Total Branched Chain Amino Acids (g/d)	17	17	17	-
Total Fiber (g/d)	55±10	28±5	42	17
Soluble	12±2	6±2	9	-
Insoluble	29±5	14±2.5	22	-

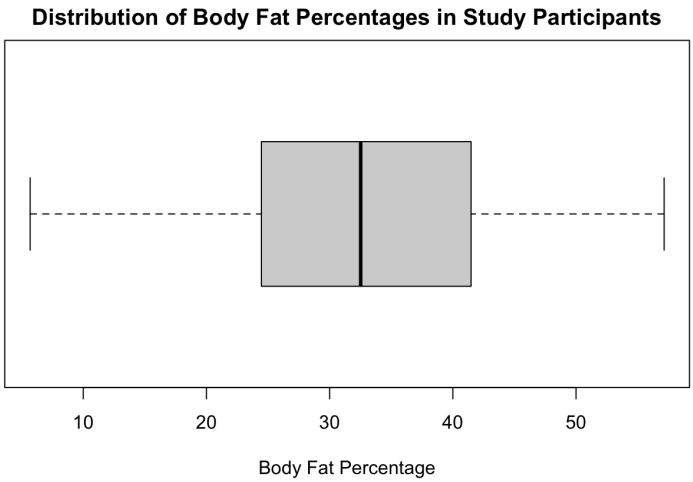

Figure 1. Distribution of body fat percentage (bf%) among study participants (n=80). Minimum bf% was 5.7, 1st quartile bf% was 24.5%, median bf% was 32.5, 3rd quartile bf% was 41.5, and maximum bf% was 57.2.

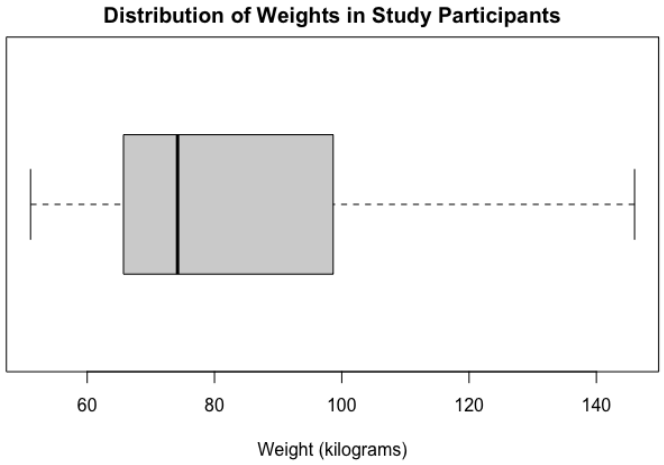

Figure 2. Distribution of weights among study participants (n=80). Minimum weight was 51.5 kg, 1st quartile weight was 65.9 kg, median weight was 74.2 kg, 3rd quartile weight was 81.1 kg, and maximum weight was 146 kg.

Associations between adiposity and concentrations of key plasma metabolites and proteins linked to BCAA catabolism

The following amino acids and BCAA catabolites were compared with body fat percentage: Leucine, isoleucine, valine, alanine, glutamic acid, propionate, succinate, malonic acid, pyruvate, ketoglutaric acid, acylcarnitine, inositol, isovaleric acid, hydroxy isovaleric acid, methylmalonate, and aminoisobutyrate. Of these, leucine (p=0.043), isoleucine (p=0.035), valine (p=0.034), pyruvate (p=0.015), alanine ($p=6e^{-4}$), glutamic acid ($p=8e^{-7}$), and isovaleric acid ($p=2e^{-}4$) were all significantly positively associated with body fat percentage. Conversely, malonic acid concentration was found to be inversely associated with body fat percentage (p=0.014) (Table 4).

Table 4. Associations between body fat percentage and metabolites related to BCAA catabolism.

Exposure Variable	**Estimate**	**SE**	**P-value** *Significance (p=0.05)*
Leucine	0.088	0.042	0.043
Isoleucine	0.117	0.054	0.035
Valine	0.112	0.052	0.034
Propionate	-0.081	0.099	0.420
Succinate	-0.106	0.062	0.095
Malonic acid	-0.541	0.215	0.014
Acetylcarnitine	-0.063	0.059	0.290
Isovaleric Acid	0.076	0.019	2.0E-04
Hydroxyisovaleric Acid	-0.066	0.104	0.532
Methylmalonate	0.017	0.035	0.633
Glutamic Acid	0.617	0.113	8.0E-07
Alanine	0.245	0.068	6.0E-04
Ketoglutaric acid	-3.3E-04	0.034	0.992
Pyruvate	0.195	0.078	0.015
Inositol	0.007	0.052	0.886

Associations between adiposity and plasma markers of inflammation and metabolism

Serum glucose, insulin, HOMA-IR score, and *hs*-CRP were all positively associated with body fat percentage ($p=2.3e^{-3}$, $7.88e^{-6}$, $3.65e^{-6}$, $5e^{-3}$, respectively). These results were significant even after adjustments for plasma leucine, isoleucine, and valine concentration and carbohydrate intake. (Table 5).

Table 5. Associations between body fat percentage and markers of inflammation and metabolism

Exposure Variable	**Estimate**	**SE**	**P-value** *Significance (p=0.05)*
hs-CRP	0.975	0.393	0.016
Glucose	0.070	0.022	2.7E-03
Insulin	0.763	0.166	2.4E-05
HOMA_IR	0.831	0.172	1.05E-05
Adiponectin	-0.258	0.150	0.091

Associations between BCAA catabolites, adipocytokines, and markers of inflammation

Adiponectin

Significant inverse correlations were observed between adiponectin concentrations and inositol (p=0.004) and hydroxy isovaleric acid (p=0.001) abundances (Table 6). Associations between adiponectin and each BCAA were not significant. No significant positive associations were observed between adiponectin and other exposure variables. Results from all metabolites in this analysis can be found in Appendix Table A.

hs-CRP

Significant positive correlations were observed between *hs*-CRP and valine, and hydroxy isovaleric acid (p=0.023 and $1.3e^{-5}$, respectively) (Table 6). There was a trend toward significant positive correlations were also observed between leucine and inositol (p=0.058 and 0.051, respectively). Results from all metabolites in this analysis can be found in Appendix Table B.

IL-6

Significant inverse associations were observed between IL-6 and alanine, and methylmalonate (0.022 and 0.014, respectively) (Table 6). There were trends toward significant positive associations between IL-6 and hydroxy isovaleric acid (p=0.055). Results from all metabolites in this analysis can be found in Appendix Table C.

Leptin

Of the adipokines and markers of inflammation included in this analysis, leptin was significantly associated with the most metabolites related to BCAA catabolism. Significant positive associations were observed between leptin and valine, leucine, isoleucine, and glutamic acid, (p=0.021, 0.042, 0.045, and 0.027, respectively). Additionally, significant inverse associations between leptin and ketoglutaric acid, acetyl carnitine, and malonic acid were also

observed (p=0.029, 0.004, and 3.07 e^{-7}, respectively) (Table 6). Results from all metabolites in this analysis can be found in Appendix Table D.

SAA

A significant positive correlation was observed between SAA and hydroxy isovaleric acid (p=0.003) (Table 6). No significant negative associations between SAA and metabolites related to BCAA catabolism were identified. Results from all metabolites in this analysis can be found in Appendix Table E.

Table 6. Associations between metabolites related to BCAA catabolism, adipocytokines, and markers of inflammation.

Outcome Variable	**Exposure Variable**	**Estimate**	**SE**	**P-value** *Significance (p=0.05)*
Adiponectin	Inositol	-0.350	±0.119	0.004
	Hydroxy-isovaleric acid	-0.240	±0.075	0.002
hs-CRP	Valine	1.530	±0.666	0.023
	Hydroxy-isovaleric acid	1.464	±0.324	1.36E-05
IL-6	Alanine	-0.427	±0.184	0.022
	Methylmalonate	-0.844	±0.338	0.014
Leptin	Valine	0.881	±0.377	0.021
	Leucine	0.892	±0.434	0.042
	Isoleucine	0.686	±0.339	0.045
	Ketoglutaric acid	-0.964	±0.435	0.029
	Acetyl carnitine	-0.443	±0.150	0.004
	Malonic acid	-0.352	±0.065	3.07E-07
	Glutamic acid	0.380	±0.170	0.027
SAA	Hydroxy-isovaleric acid	0.719	0.239	0.003

CHAPTER FIVE: DISCUSSION

Our goal in this analysis was to try to understand why BCAA are elevated among individuals with higher adiposity and insulin resistance. In order to come to a clearer conclusion, we examined the role of BCAAs in relation to adiposity and markers of metabolism, i.e., insulin resistance, adiponectin, and leptin, and inflammation, i.e., *hs*-CRP, IL-6, and SAA, in a cohort of healthy adults on controlled diets.

Three analyses were conducted in tandem to elucidate these questions. Through aim one we observed positive associations between adiposity and each BCAA, and isovaleric acid, glutamic acid, alanine, and pyruvate. These results support the hypothesis that BCAAs are part of a metabolic signature of obesity and that reductions in BCAA catabolism at the first two steps of BCAA catabolism likely drive elevations in plasma BCAAs. Aim two was able to build off these findings. Positive associations were observed between adiposity and HOMA-IR score, and *hs*-CRP even after adjusting for plasma BCAA abundance. These findings are likely explained by the role of adiposity in metabolic regulation in the context of healthy adults. In aim three, we tested the associations between BCAA catabolites and measures of metabolism and inflammation. Here we found that leptin exhibited several associations with BCAA catabolites that may converge on insulin resistance and fatty acid metabolism. These associations and associations with adiponectin and markers of inflammation should be elucidated further in future studies. These observations are further delineated below.

Associations between adiposity and key plasma metabolites linked to BCAA catabolism

As expected, positive associations were observed between body fat percentage and each BCAA. These findings are supported by observations from several clinical trials [13,21,27,38,64,75,76]

and provide further evidence that BCAAs are part of a metabolic signature associated with obesity. These observations also suggest that reductions in BCAA catabolism are driving plasma elevations of BCAAs, and these reductions are likely occurring in steps one or two of BCAA catabolism. BCAAs are essential amino acids that are almost entirely derived from diet. Although there is an additional source and sink for BCAAs within the gut microbiome [97], the impact of this pool on plasma BCAAs is minimal compared to diet. This means that either diet or reductions in catabolism can be responsible for elevations in plasma BCAA concentrations. Participants in the present analysis consumed controlled diets where total energy, carbohydrates, fats and protein were provided in a eucaloric manner specific to individual needs.[87] Subsequently, BCAAs were not over or under consumed by participants and would not drive elevations in plasma BCAA concentrations. Additionally, BCAA consumption was adjusted for in each linear mixed model. By default, the elevations observed in plasma BCAA concentrations were likely due to reductions in BCAA catabolism. Each BCAA was elevated in this analysis, indicating that alterations in BCAA catabolism are likely occurring at a step where all BCAAs are metabolized by the same enzyme. The only two steps in which BCAAs are processed by the same enzyme are steps one and two where BCAT and BCKDH transaminate and decarboxylate each BCAA, respectively.

Given that plasma BCAA concentration was elevated in those with higher body fat percentage, it was expected that BCAA catabolites would follow an inverse of this relationship. This is in congruence with the hypothesis that BCAA catabolism is hindered in the setting of adiposity resulting in reduced plasma circulation of metabolites related to BCAA catabolism.[38,66,72–74] However, positive associations between body fat percentage and isovaleric acid, glutamic acid, alanine, and pyruvate were observed in the present analysis. These

associations have been observed in a study with 634 overweight and obese adults without diabetes conducted by Vogelzangs and colleagues.[79] It is possible that the positive associations observed by Vogelzangs and in the present study are a byproduct of increased BCAA catabolic flux at the first steps of BCAA catabolism. When comparing a range of metabolic profiles in lean versus obese participants, Newgard and colleagues theorized that elevated levels of circulating BCAAs result in a larger influx of BCAAs through their respective catabolic channels.[21] This is especially plausible given that the study population in both Vogelzangs and the present analysis consisted of metabolically healthy adults free of diabetes or cardiovascular disease. A study comparing metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO) adults found reductions in adipose tissue BCAT gene expression only in MUO adults, reductions in BCKDH gene expression in MHO, and strong reductions in adipose tissue BCKDH gene expression in MUO adults.[98] Taken together, it is likely in healthy adults associations between body fat percentage and BCAA catabolic intermediates may be indicative of early reductions in BCAA catabolism, particularly at the activity of BCKDH.

It is possible that elevations in plasma BCAAs and metabolic intermediates could contribute to the development of insulin resistance in MHO adults through the glucogenic effects of alanine. BCAA catabolism provides an essential nitrogen source to produce glutamate and subsequently alanine (Figure 3). When BCAAs are deaminated to α-keto acids via BCAT activity, they produce glutamate from α-ketoglutarate.[10] Elevated plasma BCAAs may result in elevated levels of glutamate and a subsequent uptick in transamination of glutamate to alanine using pyruvate. In the present analysis, pyruvate, alanine, and glutamic acid were positively associated with body fat percentage. Alanine is considered a glucogenic amino acid in the liver as its conversion into glutamate via alanine aminotransferase (ALT), yields pyruvate which can

be used to generate glucose via gluconeogenesis. ALT has been positively associated with insulin resistance [99], and increases in alanine concentration could further contribute to the development of insulin resistance. It is important to acknowledge that most of the BCAA catabolites included in this study were downstream from BCAT and BCKDH activity. Evaluating catabolites closer, stepwise, to these enzymes, may paint a more accurate picture of BCAA catabolic activity.

The inverse association between adiposity and malonic acid may be related to the role of malonyl-CoA in fatty acid metabolism. Malonic acid-derived malonyl-CoA serves as a rate-limiting intermediate in fatty acid synthesis and an inhibitor of fatty acid oxidation.[100] Elevated free fatty acids have been observed in obese individuals, and these elevations are likely due to both increased fat mass in adipocytes and alterations in fatty acid metabolism associated with both obesity and the development of insulin resistance.[101] Additionally, a study conducted by Guiu-Jarado and colleagues comparing women with a normal BMI (<25 kg/m^2) and a obese BMI (30-38kg/m^2) concluded that subcutaneous fatty acid biosynthesis was significantly downregulated in the obese BMI cohort compared to the normal BMI cohort.[102] Therefore, malonic acid concentrations would be expected to be higher in those with lower body fat percentage and lower in those with higher body fat percentage.

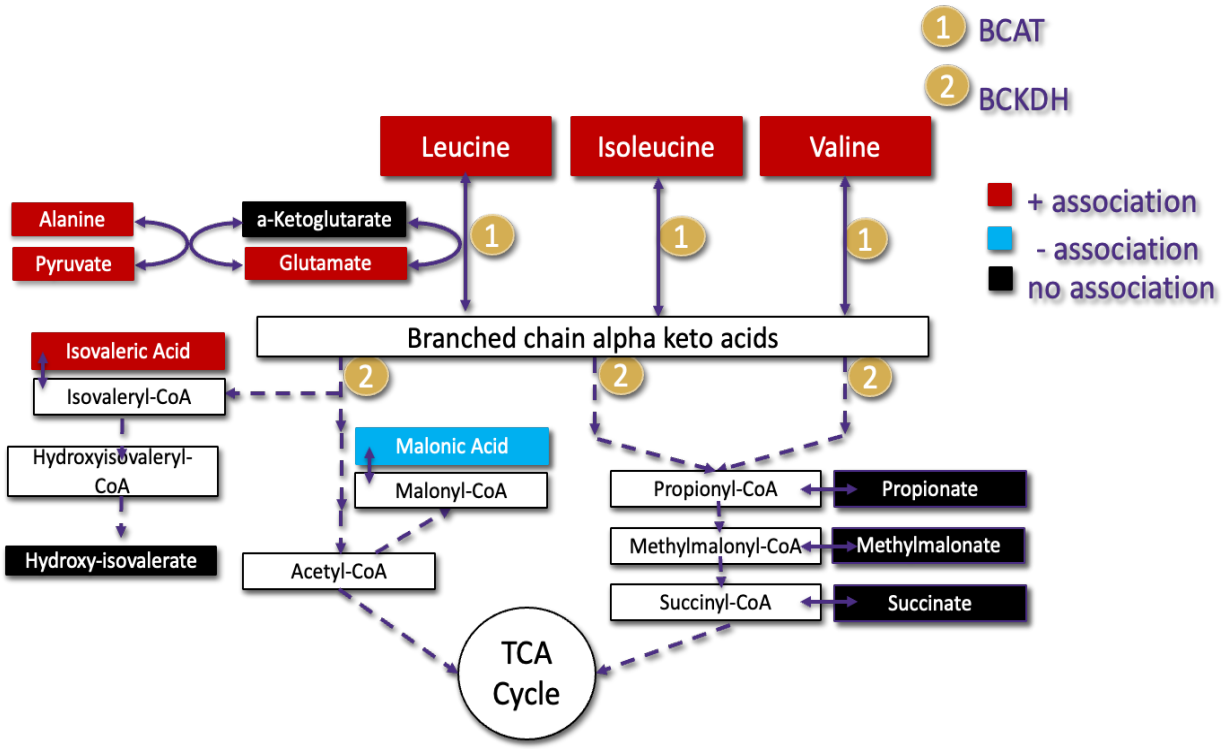

Figure 3. An abbreviated degradation pathway for leucine, isoleucine, and valine. Numbers 1 and 2 indicate the location of BCAT and BCKDH activity (respectively) within the BCAA catabolic pathway. Red indicates positive associations, blue indicates inverse associations, and black indicates no association. White boxes are branched chain a-keto acids and CoA derivatives that were not included in the metabolomics dataset of this analysis but were included in the pathway for clarity. Solid arrows indicate one step, dotted arrows indicate multiple unpictured catabolic steps.

Associations between adiposity and plasma markers of inflammation and metabolism

The significant positive associations observed between body fat percentage and markers of inflammation and metabolism align with literature that connects adiposity with chronic inflammation and insulin resistance.[30,66,83,103–105] Notably, these results were significant even after adjusting for plasma BCAA concentration and carbohydrate intake.

Obesity results in increased release of non-esterified fatty acids (NEFAs) and pro-inflammatory cytokines that are key to the development of systemic inflammation and insulin resistance. An overexpression of these proinflammatory cytokines, particularly IL-6 can lead to the stimulation of hepatocytes to secrete CRP [103], a proinflammatory marker that was positively associated with adiposity in the present study. Increased proinflammatory markers in circulation have been shown to reduce concentrations of adiponectin.[106] An inverse association was observed between body fat percentage and adiponectin, but these results were not significant.

Insulin resistance has been shown to develop in humans within hours of increased plasma NEFAs.[107] Elevated plasma NEFAs are thought to compete with glucose for substrate oxidation, leading to the recurring inhibition of pyruvate dehydrogenase, phosphofructokinase, and hexokinase II activity.[108] This results in elevated plasma glucose levels and subsequent insulin resistance. A more recent hypothesis indicates increased intracellular NEFAs may result in decreased metabolism of fatty acids, resulting in the buildup of fatty acid intermediates, including diacylglycerol, fatty acyl CoA's or ceramides.[109] These fatty acid metabolites activate a serine/threonine kinase cascade, resulting in phosphorylation of serine/threonine sites on the insulin receptor substrate-1 and 2 (IRS1/2). Serine-phosphorylated proteins from IRS1/2 fail to associate and activate phosphatidylinositol-3-OH-kinase (PI3K), a key regulator of glucose uptake in adipose tissue, muscle cells, and the liver. This results in decreased activation of

glucose transport and other downstream events related to metabolism, supporting insulin resistance development.[109] Increased release of tumor necrosis factor alpha (TNF-a), IL-6, monocyte chemoattractant protein-1 (MCP-1) and additional macrophage products also play a role in the development of insulin resistance.[110] Both TNF-a and IL-6 are thought to stimulate the c-Jun amino-terminal kinase and the IkB kinase-beta /nuclear factor-kB pathways, resulting in upregulators of inflammatory mediators that can lead to insulin resistance.[83]

In the CARB study, Navarro and colleagues observed associations between BCAA abundances and HOMA-IR scores in both low and high adiposity groups after the 28-day consumption of a high-glycemic load diet. Although these results loosely contradict the results observed in the present analysis, findings from Navarro's work are more indicative of the effects of high glycemic load diets on plasma BCAA abundance, rather than the impact of body fat percentage on plasma BCAA abundance. In the present analysis, association between body fat percentage and metabolic/inflammatory markers remained significant after adjustment for plasma BCAA abundance, and it is possible that the use of healthy participants contributed to these observations. All participants did not exhibit clinically detectable forms of metabolic dysfunction during study participation and ate a controlled amount of BCAA-containing protein specific to their needs. This scenario likely results in minimal elevation of plasma BCAAs beyond clinical norms. Additionally, the positive associations observed in the present study between plasma BCAAs and body fat percentage are not indicative of the magnitude of plasma BCAA elevation. Instead, they demonstrate the abundance of BCAAs in plasma relative to the 121 other metabolites measured in Navarro's targeted metabolomic analysis rather than the absolute concentration of plasma BCAAs. Therefore, relative to other metabolites, BCAAs may be significantly more abundant in those with a higher body fat percentage than a lower body fat

percentage, but no conclusions can be formed on how elevated plasma concentrations of BCAAs are in these groups.

Despite the observations of the present study, BCAAs have been linked by several different methods to insulin resistance development. Several review articles [28,29,42–44,49] suggest a central role of mammalian target of rapamycin complex 1 (mTORC1)/p70s6 kinase (S61k) and insulin receptor substrate 1 serine/tyrosine (IRS1 Ser/Tyr) in these mechanisms. A clinical trial conducted by Tremblay and colleagues used skeletal muscle biopsies of healthy males taken before and after postprandial peripheral insulinemia induced by a somatostatin insulin-glucose clamp.[46] Compared to saline infusions, infusion of amino acids significantly increased the activity of S6K1 and subsequent serine phosphorylation of IRS1 (Ser312). These findings are further supported by various mice experiments that were able to demonstrate a cascade effect of elevated phosphorylation of mTORC1. Elevated mTORC1 phosphorylation led to serine phosphorylation of S6k1 and subsequently, IRS1 which reduced the number of insulin-responsive receptors, predominantly in skeletal muscle.[47,48,111,112] This ultimately resulted in the development of insulin resistance derived from amino acids.

Taken together, these results highlight the role of adiposity in metabolism and inflammation, particularly in the context of healthy adults. Although several pathways converging on mTORC1 and IRS1/2 activity have been proposed as mechanisms for BCAAs to modulate metabolism and induce insulin resistance, it is likely that these pathways are only engaged in the setting of clinically significant elevations in plasma BCAA concentrations.

Associations between BCAA catabolites adipokines and markers of inflammation

This series of analyses offered a novel comparison of BCAAs and related catabolites to adipokines and markers of inflammation with the goal of identifying ways in which differences in BCAA catabolism could impact insulin resistance.

Leptin and BCAAs

Many catabolites, and all BCAAs in this analysis were associated with plasma leptin concentrations, even after adjusting for body fat percentage. The association between plasma BCAAs and leptin was expected and in-line with studies in both rats and humans. Lynch et al identified leucine as a key regulator for postprandial rises in leptin in Sprague Dawley rats [49], and these findings have been confirmed in another study in rats that determine leucine supplementation improved leptin sensitivity in the hypothalamus and adipose tissue.[113] Although much of the existing literature focuses on leucine, one clinical trial conducted in healthy Japanese adults observed positive associations between leptin and leucine, isoleucine, and valine.[114]

Leptin has multi-faceted connections to insulin resistance. Leptin-mediated signaling through the AMP-activated protein kinase pathway works to prevent insulin resistance by inhibiting pathways that antagonize insulin signaling, ultimately increasing whole-body insulin delivery and subsequently increasing insulin sensitivity.[115] Plasma leptin concentrations have also been correlated with insulin resistance in participants with normal glucose levels and those with diabetes.[116,117]

Interestingly, the observed association between BCAAs and leptin have possible connections to plasma insulin resistance. Leucine has been shown to increase leptin translation through the activity of mTORC1, the same kinase believed to be involved in the perpetuation of

insulin resistance by BCAAs.[49] Additionally, leptin resistance has been associated with insulin resistance, and it is possible that leptin resistance is perpetuated by the activity of elevated BCAAs on adipose tissue. Further studies would need to be conducted to demonstrate this association.

Leptin resistance is tightly linked with obesity, so it is also possible that the relationship between BCAAs and leptin is impacted by the residual confounding of body fat percentage. Both BCAAs and leptin are elevated in obese adults [76,115] , indicating adiposity plays a role in mediating these signaling molecules. This connection would likely become evident as adiposity increases.

Leptin and glutamic acid

Connections between leptin and glutamic acid are complicated and may be related to the central nervous system (CNS) or activity of BCAAs on insulin secretion from pancreatic beta cells. Glutamic acid is the conjugate acid of glutamate, a major excitatory neurotransmitter in the CNS. Extracellularly, leptin has been shown in mice to modify astrocyte-specific glutamate and glucose receptors in rats [118], indicating there may be a neuronal regulatory relationship between these compounds. Additionally, artificial depletion of leptin receptors (LepR) via genetic mutation in mice models resulted in lower glutamate uptake efficiency by glutamate transporter 1(GLT-1) at the hippocampus, further indicating that expression of leptin receptors at astrocytes are related to glutamate homeostasis.[119] Therefore, it is possible that rises in leptin concentrations are associated with elevated glutamate by way of reduced LepR receptors and subsequently reduced glutamate uptake receptors. Unfortunately, there are no studies conducted in humans, particularly healthy adults, to extrapolate these conclusions to the present study population.

It is also worth noting that intercellular connections between glutamate and leptin are linked to BCAA and insulin. In clonal pancreatic beta cells, leucine has been shown to activate the enzyme glutamate dehydrogenase (GDH).[120] Glutamate dehydrogenase catalyzes the interconversion of glutamate to alpha-ketoglutarate.[121] The production of glutamate is energetically preferred over production of alpha-ketoglutarate.[121] High intracellular concentrations of glutamate coupled with the depolarization effects of ATP and calcium on K^+-ATP and voltage-gated calcium channels, respectively, result in the exocytosis of insulin from beta cells. This means leucine is indirectly involved in the release of insulin via intracellular glutamate.

Interestingly, glutamate and leptin have also been shown to extracellularly inhibit insulin secretion. Glutamate is also released by islet cells and inhibits N-methyl-D-aspartate (NMDA) receptors on beta cells, reducing the rate of insulin secretion.[122] Leptin also exhibits strong inhibitory effects on pancreatic beta cells using the same receptors as glutamate. Leptin is thought to inhibit the activity of K+-ATP receptors, reducing the ATP/ADP ratio in the beta cells, reducing the amount of calcium in the cell, and subsequently impending insulin exocytosis.[123] Leptin supplementation in leptin-deficient mice has also been shown to increase density of NMDA receptors on pancreatic beta cells, directly resulting in further downregulation of insulin exocytosis.[119] This means that both glutamate and leptin share an inhibitory mechanism on pancreatic beta cells. Although all of these activities are happening intracellularly and extracellularly within the pancreas, it is possible that these cellular level actions could impact plasma concentrations of leptin and glutamate, particularly in the setting of developing insulin resistance.

It is also possible that elevated amounts of glutamic acid are being produced in the setting of elevated BCAAs, and this association between glutamic acid and leptin is actually shadowing the relationship between BCAAs and leptin. BCAA catabolism contributes nitrogen to the production of glutamate, and the more BCAAs being catabolized, the more glutamate, in theory, will be produced.

Leptin, malonic acid, and acetyl-carnitine

Acetyl-L-carnitine and malonic acid were also inversely associated with leptin concentrations, and this connection could be related to the role of leptin in fatty acid oxidation. Leptin has been shown to stimulate fatty acid oxidation in skeletal muscle through the disinhibition of carnitine palmitoyltransferase-1 (CPT-1) [124], the enzyme is responsible for the acylcarnitine formation from free carnitine and acyl-CoA.[125] Malonic acid is also a key regulator of fatty acid oxidation, with malonyl-CoA acting as a strong inhibitor of CPT-1 activity.[126] Therefore, elevated malonic acid concentrations may correlate with decreased leptin concentrations in healthy adults.

The inverse relationship observed between acylcarnitine and leptin in this analysis and other analyses [124,127] is difficult to explain. A study conducted in 400 healthy eight year old children observed through metabolic analysis that leptin was inversely associated with both long chain acylcarnitines and acetylcarnitine.[127] Additionally, older rats supplemented with acetyl-L-carnitine have exhibited significant decreases in serum leptin.[128] Acylcarnitine levels are expected to be positively associated with leptin, a stimulator of CPT-1. One possible explanation for this discrepancy is that the stimulatory effects of plasma leptin have a greater impact on intracellular acylcarnitine concentrations than plasma acylcarnitines.[127] Although plasma levels

of acylcarnitine seem to be dependent on intracellular acylcarnitine concentrations [129], the regulation mechanisms of extracellular acylcarnitine transporters are still elusive.

The connection between acylcarnitines and insulin resistance are complex. Recent metabolomic studies have associated obesity-induced insulin resistance with intramitochondrial dysfunction.[30,130,131] These findings are paralleled by accumulation of acylcarnitines in the mitochondria and subsequent depletion of TCA cycle intermediates.[132] Authors suggest that this mismatch in fatty acid oxidation and TCA cycle flux has contributed to incomplete fatty acid oxidation, and a buildup of acylcarnitines in the mitochondria, contributing to insulin resistance.[133] Acylcarnitines derived from BCAA catabolism, C3, C4-dicarboxylic carnitine, and C5 carnitine, have been shown to be elevated in participants with diabetes and obesity compared to controls.[30,31] However, diet during nutrition interventions can directly impact the concentration of these acylcarnitines. The present analysis did not evaluate data specific to BCAA-derived acylcarnitines, however it would be helpful to evaluate these associations in the context of a healthy feeding study.

Leptin and alpha-ketoglutaric acid

The inverse association between leptin and alpha-ketoglutaric acid, and this connection could indirectly be related to the connection between leptin and BCAAs. Increased BCAA concentrations could result in elevated glutamate production as BCAAs move through their catabolic channels. Glutamate is produced using alpha-ketoglutarate, so it would be expected for alpha-ketoglutaric acid concentrations to decline as glutamate concentrations increase.

There is also a possible connection between leptin, alpha-ketoglutaric acid, and bone mineral density. Alpha-ketoglutaric acid supplementation has been shown to decrease parathyroid hormone while increasing serum hydroxyproline, a key precursor of collagen.[134]

Leptin has been extensively linked to bone mass regulation via impacting HPA axis hormones that increase bodily tissue generation.[52] Overall, these associations remain unclear and further studies are needed to elucidate these observed connections.

Adiponectin and hydroxyisovaleric acid

Hydroxyisovaleric acid was associated with several metabolites, including adiponectin, C-reactive protein, and serum amyloid A. Hydroxyisovaleric acid, the conjugate acid of 3-hydroxyisovalerate, an intermediate of BCAA catabolism generated from leucine degradation. Much of the literature about this catabolite describes 3-hydroxyisovaleric acid as a urinary marker of subclinical biotin deficiency.[135,136] Isovaleric acid has also been discussed in the context of isovaleric acidemia, an autosomal recessive inborn error of leucine metabolism caused by isovaleryl-CoA deficiency resulting in the accumulation of isovaleric-CoA derivatives.[137] In healthy adults hydroxyisovaleric acid has been used as a dietary supplement to enhance muscle recovery after exercise [138], although it has not been shown to be effective when taken alone.[139]

The inverse correlation between hydroxyisovaleric acid and adiponectin is unclear. Although limited literature discusses associations between these two compounds, Connelly and colleagues observed no significant associations between leucine or other BCAAs and adiponectin when assessing groups with varying levels of glucose tolerance.[26] Conversely, a study conducted in Japanese adults without diabetes demonstrated a significant inverse relationship between all plasma BCAAs and plasma adiponectin concentrations.[114] Additionally, our multivariate analysis revealed significant associations between plasma sample batch number nine, hydroxyisovaleric acid, and adiponectin. It is possible that residual confounding from samples in batch nine impacted these results.

Adiponectin and inositol

Inositol was also inversely associated with adiponectin concentration, which is contradictory to the expected association between these compounds. Both low and high glycemic load diets that study participants consumed contained inositol which is typically found in whole grains and other fiber-containing foods. Compared to 2017-2018 NHANES data, daily fiber intake in both diet intentions was higher than the average American.[96] Inositol concentrations may be representative of a whole grain-rich diet that is inversely associated with metabolic dysregulation. Furthermore, inositol dysregulation has been associated with high glucose diets and/or altered glucose metabolism.[140] Reduced concentrations of adiponectin have been associated with metabolic dysregulation [53], and fiber intake is associated with higher concentrations of plasma adiponectin.[141] It would, therefore, be expected to observe a positive correlation between inositol and adiponectin. Further studies are needed to evaluate these associations.

Markers of inflammation

It was initially hypothesized that associations with elevated BCAAs and pro-inflammatory cytokine IL-6 may influence the relationship BCAAs have with downstream inflammatory markers regulated by IL-6, including *hs*-CRP and SAA. Although valine was positively associated with *hs*-CRP and hydroxyisovaleric acid, the associations observed between CRP, IL-6, and SAA were not congruent.

Elevated concentrations of BCAAs have been positively associated with pro-inflammatory cytokines in previous studies.[47] Additionally, results from metabolic profiling of chronic, low-grade inflammation in healthy adults identified positive associations between BCAAs and plasma CRP levels.[142] Given the regulator effects of IL-6 on CRP and SAA, it was

surprising that IL-6 did not share significant catabolite correlations with CRP and SAA in this study. In the context of healthy adults, the majority of study participants likely have normal plasma BCAA concentrations and normal concentrations of pro-inflammatory cytokines. These findings indicate that in healthy adults BCAAs are likely not playing a significant role in regulation of inflammation markers. It is possible that these associations would differ in populations with established insulin resistance.

The correlation between alanine and IL-6 is more complicated to explain. Alanine and glutamine concentrations are significantly reduced during trauma due to elevated protein catabolism.[143] Additionally, IL-6 and other proinflammatory cytokines are elevated in critical care and trauma patients.[144] There are very few studies that describe the relationship between alanine and IL-6 in non-critical care settings. One trial using whole blood of healthy human volunteers observed that incubation with L-alanine upregulated IL-6 expression in LPS-treated monocytes.[145] This evidence is contradictory to the inverse relationship observed between alanine and IL-6 in this study. Further studies are needed to evaluate these associations.

Interestingly, elevated levels of 3-hydroxyisovaleric acid and alpha-hydroxyisovaleric acid were identified in COVID-19 patients as disease severity increased from mild to severe.[146] These associations were also correlated with oxygen saturation. During hypoxia, ATP is produced through anaerobic glycolysis which is a low yield method of generating ATP. Sustained utilization of glycolytic pathways may also result in reduced NAD pools, and these pools need to be preserved to continue ATP production. Authors hypothesized that BCAA catabolism may be altered in the setting of hypoxia in order to replenish depleting nicotinamide dinucleotide (NAD) levels.[146] Although these findings are not linked to the study population in

this analysis, they shed light on the importance BCAA catabolite activity and the regulatory role of these amino acids.

STRENGTHS AND LIMITATIONS

This analysis was conducted in the context of a controlled feeding study which allowed for the impact of BCAAs to be assessed without the cofounder of increased dietary intake. Additionally, each diet part of the controlled feeding study was matched for caloric, carbohydrate, protein, and fat content, so intake amounts did not change significantly between each diet. Diets were followed for a 28 day period resembling a dietary pattern, and plasma BCAA concentrations have been more strongly correlated with habitual intake than current intake. Overall, the use of a controlled feeding study reduced noise associated with participant variations in dietary intake. Gold standard protocols were used to evaluate body fat percentage and to generate plasma metabolomic profiles for each participant. This study is the first to evaluate the associations between metabolites related to BCAA catabolism, adiposity, adipokines, and markers of inflammation. This was also the first study to correlate leptin with BCAA catabolic intermediates.

There were some limitations of this study that deserve further discussion. This analysis was cross-sectional which only allows for associations to be evaluated for a single time point. Additionally, no adjustments were made for multiple comparisons which may have contributed to false positive results. Nonetheless, many associations were very robust and provide hypotheses which can be further tested in other cohorts. All metabolomic data is generated as relative abundances which make it difficult to compare across other studies. Finally, although an extensive literature review was used to extrapolate the mechanisms behind the associations observed, we were unable to directly evaluate the role of key enzymes, particularly BCAT and BCKDH, in this analysis.

CONCLUSIONS AND FUTURE DIRECTIONS

In this cross-sectional analysis, we hypothesized that BCAAs would be elevated among individuals with higher adiposity, and be associated with insulin resistance. We found that BCAAs were elevated, likely due to aberrations in BCAA catabolism, and were correlated with adiposity, even among healthy individuals. Interestingly, leptin may be involved in this process. We speculate that mechanisms of BCAAs and adiposity converge on the regulatory effects of mTORC1 and AMP kinase. However, we were unable to directly verify this given the cross-sectional nature of this analysis. Nonetheless, this study provides data and literary support for the idea that elevated plasma BCAAs are part of a metabolic signature of obesity and reduced BCAA catabolism likely drives these elevations. Additionally, BCAAs and adiposity are influential in the development of insulin resistance, even in the context of healthy adults. Overall, this study describes the complex signalling effects of BCAAs, and results from this study sets the groundwork for future analyses to further explore these associations.

Future studies should incorporate proteomic and lipidomic datasets from the CARB study in order to evaluate other components of the BCAA catabolic pathway. The inclusion of 3-hydroxyisobutyrate, acetoacetate, and C3/C5 acylcarnitine would also be recommended. 3-hydroxyisobutyrate, acetoacetate, and C3/C5 aclycarninte are BCAA catabolites that arise after BCKDH activity. These metabolites serve as key representors of BCAA catabolism in previous literature and their inclusion would provide strong evidence to delineate whether reductions in BCAT or BCKDH activity contribute to elevated plasma BCAA concentrations. Performing the present analysis with stratifications by sex, body fat percentage, and low/high glycemic load diet would be helpful to further understand how these confounders impact plasma BCAA

concnetration. Finally, it would be interesting to explore the role of the gut microbiome in these associations as gut microbiomes are a potential source and sink for BCAAs.

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APPENDIX

Table A. Association between plasma adiponectin concentration and metabolites related to BCAA catabolism.

Exposure Variable	**Estimate**	**SE**	**P-value** *Significance (p=0.05)*
Valine	-0.128	±0.152	0.402
Leucine	-0.130	±0.169	0.443
Isoleucine	-0.165	±0.131	0.210
Propionate	-0.034	±0.051	0.503
Pyruvate	-0.037	±0.057	0.512
Succinate	0.084	±0.077	0.281
Ketoglutaric acid	0.248	±0.156	0.116
Acetyl carnitine	-0.054	±0.054	0.322
Malonic acid	-0.021	±0.024	0.395
Alanine	-0.052	±0.079	0.515
Inositol	-0.350	±0.119	0.004
Hydroxy-isovaleric acid	-0.240	±0.075	0.002
Methylmalonate	0.038	±0.146	0.793
Isovaleric acid	-0.302	±0.246	0.223
Glutamic acid	-0.084	±0.066	0.203

Table B. Association between plasma *hs*-CRP concentration and metabolites related to BCAA catabolism.

Exposure Variable	**Estimate**	**SE**	**P-value** *Significance (p=0.05)*
Valine	1.530	±0.666	0.023
Leucine	1.463	±0.766	0.058
Isoleucine	1.017	±0.600	0.092
Propionate	0.271	±0.257	0.293
Pyruvate	-0.224	±0.292	0.444
Succinate	0.132	±0.393	0.738
Ketoglutaric acid	-1.161	±0.777	0.138
Acetyl carnitine	0.058	±0.270	0.830
Malonic acid	-0.059	±0.123	0.634
Alanine	-0.507	±0.391	0.197
Inositol	1.133	±0.577	0.052
Hydroxy-isovaleric acid	1.464	±0.324	1.36E-05
Methylmalonate	-0.623	±0.734	0.398
Isovaleric acid	0.495	±1.273	0.698
Glutamic acid	0.092	±0.306	0.765

Table C. Association between plasma IL-6 concentration and metabolites related to BCAA catabolism.

Exposure	**Estimate**	**SE**	**P-value** *Significance (p=0.05)*
Valine	0.589	±0.330	0.076
Leucine	0.580	±0.375	0.125
Isoleucine	0.358	±0.294	0.225
Propionate	0.161	±0.122	0.188
Pyruvate	-0.196	±0.138	0.158
Succinate	-0.123	±0.187	0.511
Ketoglutaric acid	-0.322	±0.372	0.389
Acetyl carnitine	0.055	±0.129	0.667
Malonic acid	0.006	±0.058	0.925
Alanine	-0.427	±0.184	0.022
Inositol	0.485	±0.280	0.086
Hydroxy-isovaleric acid	0.327	±0.169	0.055
Methylmalonate	-0.844	±0.338	0.014
Isovaleric acid	0.225	±0.602	0.709
Glutamic acid	-0.158	±0.148	0.286

Table D. Association between plasma leptin concentration and metabolites related to BCAA catabolism.

Exposure	**Estimate**	**SE**	**P-value** *Significance (p=0.05)*
Valine	0.881	±0.377	0.021
Leucine	0.892	±0.434	0.042
Isoleucine	0.686	±0.339	0.045
Propionate	-0.119	±0.146	0.414
Pyruvate	-0.080	±0.165	0.628
Succinate	-0.226	±0.222	0.311
Ketoglutaric acid	-0.964	±0.435	0.029
Acetyl carnitine	-0.443	±0.150	0.004
Malonic acid	-0.352	±0.065	3.07E-07
Alanine	-0.082	±0.223	0.714
Inositol	0.120	±0.331	0.718
Hydroxy-isovaleric acid	0.092	±0.197	0.641
Methylmalonate	-0.083	±0.416	0.843
Isovaleric acid	-0.514	±0.716	0.474
Glutamic acid	0.380	±0.170	0.027

Table E. Association between plasma SAA concentration and metabolites related to BCAA catabolism

Exposure	**Estimate**	**SE**	**P-value** *Significance (p=0.05)*
Valine	0.503	0.484	0.301
Leucine	0.709	0.556	0.204
Isoleucine	0.315	0.435	0.471
Propionate	0.100	0.192	0.603
Pyruvate	-0.194	0.219	0.378
Succinate	0.189	0.293	0.520
Ketoglutaric acid	-0.371	0.580	0.523
Acetyl carnitine	-0.020	0.200	0.919
Malonic acid	-0.047	0.091	0.605
Alanine	-0.138	0.292	0.638
Inositol	0.416	0.422	0.326
Hydroxy-isovaleric acid	0.719	0.239	0.003
Methylmalonate	-0.447	0.547	0.415
Isovaleric acid	-0.412	0.950	0.665
Glutamic acid	-0.029	0.221	0.897