Whey Protein Supplementation Does Not Alter Plasma Branched-Chained Amino Acid Profiles but Results in Unique Metabolomics Patterns in Obese Women Enrolled in an 8-Week Weight Loss Trial1–4

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Abstract

Background: It has been suggested that perturbations in branched-chain amino acid (BCAA) catabolism are associated with insulin resistance and contribute to elevated systemic BCAAs. Evidence in rodents suggests dietary protein rich in BCAAs can increase BCAA catabolism, but there is limited evidence in humans.

Objective: We hypothesize that a diet rich in BCAAs will increase BCAA catabolism, which will manifest in a reduction of fasting plasma BCAA concentrations.

Methods: The metabolome of 27 obese women with metabolic syndrome before and after weight loss was investigated to identify changes in BCAA metabolism using GC-time-of-flight mass spectrometry. Subjects were enrolled in an 8-wk weight-loss study including either a 20-g/d whey (whey group, n = 16) or gelatin (gelatin group, n = 11) protein supplement. When matched for total protein by weight, whey protein has 3 times the amount of BCAAs compared with gelatin protein.

Results: Postintervention plasma abundances of Ile (gelatin group: 637 ± 18, quantifier ion peak height O100; whey group: 744 ± 65), Leu (gelatin group: 1210 ± 33; whey group: 1380 ± 79), and Val (gelatin group: 2080 ± 59; whey group: 2510 ± 230) did not differ between treatment groups. BCAAs were significantly correlated with homeostasis model assessment of insulin resistance at baseline (r = 0.52, 0.43, and 0.49 for Leu, Ile, and Val, respectively; all, P < 0.05), but correlations were no longer significant at postintervention. Pro- and Cys-related pathways were found discriminant of whey protein vs. gelatin protein supplementation in multivariate statistical analyses.

Conclusions: These findings suggest that BCAA metabolism is, at best, only modestly affected at a whey protein supplementation dose of 20 g/d. Furthermore, the loss of an association between postintervention BCAA and homeostasis model assessment suggests that factors associated with calorie restriction or protein intake affect how plasma BCAAs relate to insulin sensitivity. This trial was registered at clinicaltrials.gov as NCT00739479.

Keywords: obesity, weight loss, metabolic syndrome, dairy, protein, BCAA, Leu, metabolomics, Cys, Pro

Introduction

Elevations of plasma glucose and nonesterified FAs have been well characterized in the obese condition with insulin resistance or type 2 diabetes (T2D). In addition, concurrent rises in plasma BCAAs have also been observed (1–7). Although insulin resistance and T2D are synonymous with impaired glucose homeostasis, the etiology of these conditions is likely linked to a...
broad dysregulation of mitochondrial function and associated pathways involving both FA and amino acid metabolism. We have speculated that a reduced flow of anaplerotic carbon into the tricarboxylic acid cycle in situ contributes to the mitochondrial dysfunction often seen in the insulin-resistant state and T2D (8). A “mismatch” of anaplerotic substrate relative to loss (through cataplerosis) is hypothesized to reduce the capacity of the tricarboxylic acid cycle to accept acetyl-CoA, which further impairs the mitochondria’s ability to efficiently use FAs (8). The hypothesis that an impairment of complete BCAA catabolism, in part through reduced mitochondrial branched-chain ketoacid dehydrogenase complex (BCKDC) activity, contributes to these outcomes and observed elevations in blood BCAAs with insulin resistance was further elaborated (9). Our working hypothesis regarding elevated blood BCAAs in the insulin-resistant or T2D state is that mitochondrial BCKDC activity, an irreversible metabolic step in BCAA oxidative catabolism considered to be rate-limiting, is attenuated in its expression and/or activity under these conditions, at least in some tissues (9, 10). The evidence supporting altered BCKDC expression in the insulin-resistant state has been derived from both animal and human studies. Regarding the latter, in bariatric surgery patients, postsurgery reductions in plasma BCAAs were observed concurrently with elevation of total BCKDC content in subcutaneous and visceral adipose biopsy specimens (11). In obese women with insulin resistance and metabolic syndrome, BCKDC component gene expression was significantly lower in visceral adipose tissue compared with equally obese healthy controls (11). Additionally, transcript abundances for subcutaneous adipose tissue BCKDC components have been positively correlated to indices of insulin sensitivity in 2 separate studies (12, 13), indicating that BCKDC expression is lower in human white adipose tissue with impaired glucose homeostasis. Taken together, these observations support the idea that a reduction in complete, efficient mitochondrial BCAA oxidation, partly through lower BCAA catabolic enzyme expression and activity in some tissue, is a characteristic of the insulin-resistant and T2D pathophenotypes.

The activity of hepatic BCKDC has long been known to be sensitive to dietary inputs in animal models. Rodent studies established that hepatic BCKDC activity is greatly diminished with low-protein diets (14–16), likely as an adaptation that conserves essential amino acids for their various regulatory functions (14). Conversely, introduction of higher dietary protein intake or Leu administration in previously protein-deficient rodents significantly increases BCKDC activity in rodents (16–18). In a study investigating the effects of dietary nitrogen intake on male Sprague-Dawley rats, rats fed a diet containing casein protein or a protein mix composed of soybean, meat, fish, and milk powder had higher BCKDC activity than rats fed diets composed of isolated amino acids or partial casein hydrolysate (19). These lines of evidence suggest that in addition to the total dietary protein amount, the type and quality of dietary protein affect BCKDC activity. Applying this research to human subjects is a challenge considering the difficulty of procuring multitemplate biopsy specimens under well-controlled conditions of dietary protein modulation. As an alternative approach to the problem, the present study leveraged metabolomics to assess differences in plasma metabolites from obese women consuming gelatin vs. whey protein supplements in an 8-wk weight-loss trial (20). Whey and gelatin were chosen for this particular weight-loss study because of their differences in essential amino acids: whey is a complete protein and gelatin is an incomplete protein in regard to their essential amino acid profile and, when matched for total protein by gram weight, whey protein has 3 times the amount of BCAAs compared with gelatin protein (21). Thus, we hypothesized that the increased daily BCAA load with a whey protein–supplemented diet would elicit adaptive increased tissue BCKDC expression, as reflected in lower fasting plasma BCAA and branched-chain keto acid (BCKA) metabolites. In addition to examining BCAA patterns in the cohort, we took advantage of comprehensive untargeted metabolomics to further understand how changes in dietary protein source affect multiple metabolic pathways in humans. To our knowledge, these approaches have not been conducted previously to examine how specific dietary proteins affect BCAA metabolism in a clinical setting.

Methods

Subjects
Details of subject recruitment, subject characteristics, and diets have been described previously (20). Briefly, 41 obese adults with metabolic syndrome from the greater Sacramento region were enrolled for participation in this study. Metabolic syndrome was diagnosed using the current diagnostic criteria according to the National Cholesterol Education Program’s Adult Treatment Panel III. Of the 41 subjects enrolled, 35 completed the 8-wk intervention (29 women, 6 men). To minimize potential sex-associated variance in metabolomics patterns, only female participants were studied in the current experiment. Exclusion criteria were T2D; recent weight change; pregnancy or lactation; tobacco use; active eating, digestive, or absorptive disorders; use of herbal or protein supplements; or use of certain medications such as insulin sensitizers, statins, and/or angiotensin-converting enzyme inhibitors.

Design
This study was an 8-wk double-blinded, placebo-controlled, randomized weight-loss intervention where participants were randomly assigned into 2 parallel treatment groups receiving 2 daily doses of either a 10-g/dose placebo gelatin-based protein supplement (Glanbia, Inc.) or a 10-g/dose whey-based protein supplement (Glanbia, Inc.). Thus, all subjects received a total of 20 g/d of the respective protein supplements in addition to their weight-loss–targeted diets. All participants provided informed consent as approved by the Institutional Review Board of the University of California at Davis. The trial was registered at clinicaltrials.gov (NCT00739479).

Diets
All subjects consumed an energy-restricted diet for 8 wk based on ideal body weight-for-height calculations with a primary aim of a 5–10% reduction in total body weight. Subjects received diet counseling and targeted a diet (including supplemental protein) consisting of 17% protein, 53% carbohydrates, and 30% fat. Similar diet plans were followed in both groups and contained both animal and plant protein–based foods. Macronutrient content of diet was assessed with Food Processor SQL, version 9.8 (Esha Research). Gelatin and whey protein supplements were packaged in identical-appearing containers in 10-g portions containing 40 kcal/package; participants were instructed to consume the doses with 8 oz of water 30 min before breakfast and again 30 min before lunch. The supplements had identical flavorings to ensure blinding. Subjects were also provided instructional binders containing their meal plan, recipes, grocery lists, tips for dining out, and tips for successful dieting. In addition, diet diaries were given to subjects to record food consumption, protein supplement usage, and physical activity. Diaries were returned and used during diet counseling visits on a

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10 Abbreviations used: BB, BinBase; BCKA, branched-chain keto acid; BCKDC, branched-chain keto acid dehydrogenase complex; PCC, principal component analysis; PLS-DA, partial least-squares–discriminant analysis; T2D, type 2 diabetes; VIP, variable importance in projection.
biweekly basis. Subjects were advised to maintain their normal physical activity throughout the study.

**Anthropometric measurements**

Body weight and waist circumferences were measured preintervention and every 2 wk throughout the study at the Clinical and Translational Science Center, Clinical Research Center of the University of California at Davis. Weight was determined in light clothing and without shoes using a digital medical scale (Tanita, BWB800-P Doctors Scale; Arlington Heights, IL). Height without shoes was measured using a wall-mounted stadiometer (Ayrton Stadiometer, Model S100; Ayrton).

**Plasma handling and analyses**

Pre- and postintervention fasting blood samples were obtained between 0600 and 0900 after an overnight fast. Sodium fluoride–containing tubes were used for blood draws for glucose measurements, serum prepared for insulin, and other samples used for metabolomics analysis collected in tubes containing EDTA. Plasma and serum samples were immediately processed and stored at –80°C until analysis. Glucose was measured using a YSI 2300 STAT Plus Glucose and Lactate Analyzer (YSI Life Sciences) and serum insulin was measured using radioimmunoassay technology (Linco Research, Inc.). Insulin sensitivity was assessed by the homeostatic model assessment (HOMA-IR) using the following formula: [fasting insulin (mU/mL) · fasting glucose (mM)]/22.5. Plasma TGs, total cholesterol, and HDL cholesterol were measured using a Polyclad Polychrom immunoassay analyzer (Polymedicno, Inc.). LDL cholesterol was calculated based on the following equation: total cholesterol − HDL − (TG/5). Analytic performances of these measurements have been described previously (20). Plasma samples used for untargeted metabolomics analyses were extracted and derivatized by silylation/methoximation before analysis by GC-time-of-flight MS, as previously described (2).The resulting data from untargeted metabolomics analysis were processed using the BinBase (BB) database (22). BB matches the metabolic features retention index and mass spectrum information from the analysis against the Fiehn mass spectral library of 1200 authentic metabolite spectra and NIST05 commercial library. Metabolites were reported if present within at least 50% of the samples per study design group (23). Peak heights of quantifier ions for each metabolite were normalized to the sum intensities of all reported metabolites. These relative abundances were used for statistical analyses. All plasma metabolomics data presented in the manuscript, including BCAA, are reported as quantifier ion peak height ÷ 100.

**Statistics**

All analyses were conducted in R version 3.0.2 (24). Data are presented as means ± SDs in text. The α was determined at 0.05 for all statistical tests unless alternatively specified.

**Assessment of clinical and plasma BCAA indices.** ANCOVA was used to assess postintervention differences in whey vs. gelatin protein supplementation with preintervention measurements as a covariate to control for baseline. P-values obtained from ANCOVA tests were corrected for false discovery rate (25) at Q ≤ 0.05. The effect of protein supplementation on absolute changes in plasma BCAA and BCKA abundances was assessed by Mann-Whitney U tests. Associations between plasma BCAA abundances and all other variables were assessed with Spearman correlations.

**Multivariate modeling of dietary protein supplementation.** Data were first assessed for univariate outliers by Grubb’s test for outliers (26) at α = 0.01. In total, 19% of all variables contained at least 1 outlier and outliers consisted of 0.56% of all data (Supplemental Table 1). Of the variables that had outliers, only 2 had outlier prevalence ≥10%. These 2 variables (paracetamol and BB759470) were excluded from analyses. All other outliers were removed from data analysis and imputed using a singular value decomposition–based method (27) from the “imputation” package (28). Imputation of outliers was conducted within treatment class. The distributions of all variables were assessed for normality with Anderson-Darling tests (29). Variables that did not have a normal distribution were transformed with either log, square root, or power transformations (Supplemental Table 1).

Partial least-squares–discriminant analysis (PLS-DA) was used to characterize whey vs. gelatin protein supplementation in postintervention samples. PLS-DA is an extension of partial least-squares regression, which is a supervised multivariate method that reduces high-dimensional data into orthogonal latent variables while maximizing the correlation between the explanatory variables and the response variable. Data pertaining to subject clustering and variables that contribute to subject clustering can be visualized by plotting scores and loadings, respectively, from PLS-DA latent variables. The classic orthogonal scores algorithm was implemented using the “pls” package with internal cross-validation (30).

An external cross-validation scheme was used to assess model performance and to reduce overfitting. Two-thirds of the subjects were randomly selected as a “training set” to develop the PLS-DA models, and the remaining one-third were used as a “test set” to determine model performance. Subjects selected as the test set are provided in Supplemental Table 1. Model assessment was based on the ability of the model fit with subjects from the training set to correctly identify the treatment assignment of subjects within the test set.

Variables that were included in final PLS-DA models were selected by variable importance in projection (VIP) rankings or through a univariate comparison of class means (e.g., ANCOVA). VIP is a weighted measure of the contribution of every predictor variable according to the variance explained in the latent variable of the PLS-DA model (31, 32). We used bootstrapping to assess the precision of VIP calculations ± 1 (31).

Alternatively, variables with a significant treatment effect were selected to be included in PLS-DA models. Treatment effects in all variables were assessed by ANCOVA as described previously.

Principal component analysis (PCA) was used to illustrate how featured variables discriminate subjects from the test set. PCA is an undirected multivariate analysis that can partition high-dimensional data into orthogonal “principal components.” Scores and loading are also produced in PCA and can be plotted to visualize any clustering of subjects (scores plot) and variables contributing to the subject clustering (loadings plot).

**Results**

**Participant’s dietary and clinical characteristics.** Twenty-nine women completed the 8-wk diet plan and were included in this study. Two additional subjects with baseline fasting glucose above 125 mg/dL, indicating potentially undiagnosed T2D, were removed from the analyses (Supplemental Table 1). The mean age of participants in this study was 41 ± 9.8 y and ranged between 18 and 56 y. Pre- and postintervention energy and macronutrient composition by gelatin and whey protein groups is provided in Table 1. On average, participants reduced their energy intake by 2740 ± 584 kJ. Actual macronutrient consumption was closer to ~20% protein, ~47% carbohydrates, and ~32% fat.

The clinical characteristics of participants by treatment assignment are provided in Table 2. As previously described, total weight, BMI, waist circumference, fasting insulin, and total TGs were all significantly reduced over the 8-wk intervention (20). Waist circumference was the only clinical parameter that was significantly different with protein supplement consumption and it was slightly lower with gelatin protein consumption (P < 0.05; Table 2).

**Data characteristics.** A total of 439 independent biologic parameters were assessed for each subject in the study, including metabolomics and clinical measurements (Supplemental Table 1). There were a total of 426 metabolites detected in the untargeted metabolomics analysis and 128 of these were annotated based on their structural identification. The remaining “unknown” metabolites that did not have full structural identification were assigned BB identification numbers (23) and were included in all statistical analyses.
BCAA metabolism. Pre- and postintervention plasma abundances of Ile, Leu, Val, and total BCAAs by protein supplementation groups are provided in Table 3. Only Leu and Val approached, but did not achieve, statistical significance (P = 0.09). Figure 1 presents the absolute changes in BCAAs and BCAA derivatives by protein supplementation groups. None were found to differ between whey or gelatin protein–supplemented groups.

Relations of plasma BCAAs abundances to other metabolites. Correlations among BCAAs and various chemical compounds were also evaluated (Figure 2). In addition to glucose metabolism indices, a broad array of metabolites were strongly associated with BCAAs at both pre- and postintervention (Figure 2). In general, BCAA metabolism and associations with other plasma metabolites were strikingly different at both pre- and postintervention. As expected, the plasma concentrations of individual BCAAs were positively correlated with one another. BCAAs were strongly associated with a suite of aromatic amino acids and their derivatives before but not after dietary intervention (Figure 2). Conversely, 4-hydroxyproline, trans-4-hydroxyproline, β-Ala, Cys, cystine, and Cys-Gly did not associate with BCAAs at preintervention but were correlated to BCAAs after the intervention (Figure 2).

All BCAAs were positively associated with HOMA-IR only at preintervention (P = 0.01, 0.02, and 0.01 for Leu, Ile, and Val, respectively; Figure 3 A). We observed a subject with an unusually

| Table 1 | Macronutrient composition of diet consumed by obese women with metabolic syndrome before and after an 8-wk weight-loss regimen including 20 g/d of dietary gelatin or whey protein supplements.1 |
| --- | --- | --- | --- | --- |
| | Gelatin | | Whey | | ANCOVA (P) |
| Total energy, kJ | | | | | |
| Pre | 8608 ± 464 | 5649 ± 433 | 7866 ± 818 | 5302 ± 292 | 0.60 |
| Protein | | | | | |
| Dietary protein | | | | | |
| g | 97 ± 14 | 49 ± 5 | 82 ± 9 | 45 ± 5 | 0.50 |
| kJ | 1620 ± 240 | 819 ± 86 | 1360 ± 130 | 756 ± 76 | 0.50 |
| % of total energy | 18.4 ± 1.9 | 14.6 ± 1.1 | 17.8 ± 1.1 | 13.7 ± 0.9 | 0.15 |
| Supplemental protein | | | | | |
| g | 0 | 20 | 0 | 20 | — |
| kJ | 0 | 335 | 0 | 335 | — |
| % of total energy | 0 | 6.21 ± 0.43 | 0 | 6.34 ± 0.34 | — |
| Total protein | | | | | |
| % of total energy | 18.4 ± 1.9 | 20.8 ± 1.2 | 17.8 ± 1.1 | 20 ± 0.9 | 0.24 |
| Carbohydrates | | | | | |
| g | 245 ± 15 | 156 ± 10 | 219 ± 23 | 157 ± 10 | 0.87 |
| kJ | 4100 ± 240 | 2610 ± 170 | 3660 ± 380 | 2630 ± 170 | 0.87 |
| % of total energy | 47.5 ± 2.0 | 47 ± 1.8 | 45.3 ± 1.1 | 48.1 ± 2.0 | 0.69 |
| Fat | | | | | |
| g | 78 ± 6 | 49 ± 5 | 79 ± 10 | 46 ± 3 | 0.66 |
| kJ | 2950 ± 210 | 1830 ± 190 | 2970 ± 380 | 1740 ± 130 | 0.66 |
| % of total energy | 34.1 ± 1.9 | 32.2 ± 1.9 | 36.3 ± 1.4 | 31.8 ± 1.8 | 0.93 |

1 Data are means ± SEMs of 3-d food records, n = 9 (gelatin) or 12 (whey). Postintervention means were compared by ANCOVA with preintervention measurements as covariates. Records were unavailable from 2 (gelatin) and 4 (whey) participants. Post, postintervention; Pre, preintervention.

| Table 2 | Clinical characteristics of obese women with metabolic syndrome before and after an 8-wk weight-loss regimen including 20 g/d of dietary gelatin or whey protein supplements.1 |
| --- | --- | --- | --- | --- |
| Variable | Gelatin | | Whey | | ANCOVA (P) |
| Weight, kg | 94.2 ± 2.8 | 87.9 ± 2.8 | 102 ± 2.6 | 96.1 ± 2.4 | 0.40 |
| BMI, kg/m² | 36.0 ± 1.2 | 33.5 ± 1.1 | 36.8 ± 0.79 | 34.6 ± 0.84 | 0.62 |
| Waist circumference, cm | 39.2 ± 1.1 | 36.2 ± 0.99 | 39.9 ± 0.74 | 37.9 ± 0.76 | 0.05 |
| Glucose, mg/dL | 96.3 ± 2.4 | 93.3 ± 2.9 | 97.8 ± 2.5 | 97.8 ± 2.5 | 0.25 |
| Insulin, µU/mL | 23.6 ± 1.8 | 19.3 ± 2.6 | 18.8 ± 1.9 | 16.3 ± 1.4 | 0.88 |
| HOMA-IR | 5.58 ± 0.43 | 4.41 ± 0.55 | 4.62 ± 0.55 | 3.94 ± 0.37 | 0.92 |
| Total TGs, mg/dL | 146 ± 19 | 123 ± 13 | 167 ± 22 | 144 ± 17 | 0.57 |
| Total cholesterol, mg/dL | 199 ± 11 | 182 ± 13 | 198 ± 6.2 | 186 ± 5.5 | 0.55 |
| LDL cholesterol, mg/dL | 122 ± 8.5 | 112 ± 9.6 | 116 ± 5.0 | 110 ± 4.5 | 0.73 |
| HDL cholesterol, mg/dL | 47.5 ± 2.9 | 45.4 ± 3.8 | 48.6 ± 2.9 | 47.1 ± 2.8 | 0.79 |

1 All measurements were conducted after an overnight fast. Blood measurements were conducted on plasma except for insulin (serum). Data are means ± SEMs, n = 11 (gelatin) or 16 (whey). Postintervention means were compared by ANCOVA with preintervention measurements as covariates. Post, postintervention; Pre, preintervention.
high value across all BCAAs compared with the other values in the postintervention condition (Figure 3B) and reassessed postintervention correlations between each BCAA and homeostasis model assessment with exclusion of the outlier. Only Val was statistically significant ($P = 0.05$) with this potential outlier removed (Supplemental Figure 1).

**Multivariate discrimination of whey vs. gelatin protein supplementation.** In addition to BCAA metabolism, it is possible that supplementation with proteins differing in composition affects broader metabolic systems. Thus, as a secondary aim we leveraged the samples to determine if specific metabolite profiles distinguish individuals consuming either a 20-g/d whey protein supplement rich in BCAAs vs. a gelatin protein supplement limited in BCAAs and other essential amino acids over an 8-wk period of energy restriction. Because measurements at preintervention were influenced by unsupervised dietary food consumption that occurred just before entering the study, we initially focused our analysis on postintervention results. Metabolites and clinical characteristics that characterized whey consumption were identified with a PLS-DA model fitted with metabolites higher in individuals consuming whey protein) feeding. Metabolite high positive loading values in model 1 (i.e., clusters by protein supplementation based on the variables selected in model 1 (Supplemental Figure 2). All discriminant amino acids, including BCAAs, identified by the PLS-DA model at postintervention were lower after whey supplementation (Figure 4). Plasma Cys and Pro derivatives appeared to be the most influential amino acids based on their larger loading values. Although plasma BCAAs did not differ by dietary protein supplementation in ANCOVA analyses, each plasma BCAA was discriminant of whey vs. gelatin consumption at the postintervention sampling point. Other discriminant amino acids and amino acid derivatives included aspartate, Ser, and β-Ala. Like the BCAAs, HOMA-IR and fasting insulin were also discriminant of protein supplement consumption in model 1 and were lower in participants under whey protein supplement feeding. Metabolite high positive loading values in model 1 (i.e., metabolites higher in individuals consuming whey protein) included fumarate, glyceric acid, cholesterol (untargeted metabolomics value), α-tocopherol, and xylitol.

Our first pass at discriminating whey consumption focused solely on postintervention measurements (model 1) and did not account for baseline differences among the groups. This may

### TABLE 3  Plasma BCAAs, 2-ketoisocaproic acid, and 2-hydroxybutanoic (2-hyroxybutryrate) concentrations in obese women with metabolic syndrome before and after an 8-wk weight-loss regimen including 20 g/d of dietary gelatin or whey protein supplements

<table>
<thead>
<tr>
<th>Variable $^3$</th>
<th>Gelatin</th>
<th>Whey</th>
<th>ANCOVA(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Ile, QIPC</td>
<td>662 ± 33</td>
<td>744 ± 65</td>
<td>581 ± 29</td>
</tr>
<tr>
<td>Leu, QIPC</td>
<td>1280 ± 43</td>
<td>1380 ± 79</td>
<td>1160 ± 63</td>
</tr>
<tr>
<td>Val, QIPC</td>
<td>2290 ± 130</td>
<td>2510 ± 230</td>
<td>2080 ± 12</td>
</tr>
<tr>
<td>Total BCAA, QIPC</td>
<td>4260 ± 190</td>
<td>4840 ± 370</td>
<td>3830 ± 210</td>
</tr>
<tr>
<td>2-Ketoisocaproic acid, QIPC</td>
<td>8.99 ± 2.0</td>
<td>6.96 ± 0.78</td>
<td>7.74 ± 1.5</td>
</tr>
<tr>
<td>2-Hydroxybutanoic acid, QIPC</td>
<td>271 ± 19</td>
<td>310 ± 23</td>
<td>271 ± 27</td>
</tr>
</tbody>
</table>

$^3$ All measurements were conducted after an overnight fast. Data are means ± SEMs, n = 11 (gelatin) or 16 (whey). Postintervention means were compared by ANCOVA with preintervention measurements as covariates. Post, postintervention; Pre, preintervention. QIPC, quantifier ion peak height. $^3$ Reported as QIPC = 100.
explain the inconsistencies between model 1 and ANCOVA analyses (e.g., Leu and other BCAAs were identified in model 1 but not statistically significant in ANCOVA analysis). We therefore selected postintervention metabolites that were significantly different by ANCOVA with baseline abundances used as covariate. Using the same 18 subjects to fit model 1, 34 metabolites were found significantly different between whey and gelatin feeding (Supplemental Table 3); these metabolites were used to develop the PLS-DA model 2 (Figure 5). Similar to model 1, model 2 only misclassified 1 subject and PCA was able to define separate clusters in test subjects (Supplemental Figure 3). All 34 metabolites used to fit model 2 were featured in model 1. Pro derivatives, Cys, cystine, isoheptadecanoic acid, fumaric acid, and cholesterol were annotated metabolites with the greatest absolute loading values in model 2. Differences in these metabolites were specifically associated with whey vs. gelatin supplementation, even after baseline adjustment. In addition, a number of unknown metabolites (nonannotated BB analytes) also contributed to separation of whey-supplemented vs. gelatin-supplemented individuals.

**Discussion**

This study investigated whether an 8-wk dietary consumption of a supplemental protein rich in BCAAs, relative to a supplemental control protein comparatively lower in BCAAs, would alter blood biomarkers of BCAA metabolism in women with metabolic syndrome. Specifically, we hypothesized that postintervention fasting abundances of blood BCAAs and BCKAs would be reduced with 20 g/d of whey protein supplementation compared with gelatin protein supplementation, based on previous studies that have observed an increase in BCKDC activity with higher BCAAs in rodents (16–18, 33, 34). However, we observed little difference in plasma BCAAs or BCKAs abundances between protein supplement groups with this feeding regimen. Plasma BCAA abundances were factors that discriminated whey vs. gelatin supplementation in multivariate statistical models, but this observation was lost in models that accounted for preintervention (baseline) blood values.

There are several possible explanations for the lack of robust association between whey supplementation and fasting blood BCAA patterns reported here. First, it is possible that supplementation would only be effective to modify BCAA-associated enzymes and metabolism under conditions in which starting protein intake is inadequate or low. Early animal work demonstrated a linear increase in hepatic BCKDC activity in rats fed diets ranging from protein deficient up to 40% casein after adaptation to low-protein diets (16, 33). In contrast to these studies, the participants in our study likely maintained adequate or above adequate protein intake before enrollment, based on current dietary protein intake in the United States (35). Second, circulating BCAA concentrations may not be sensitive enough to detect subtle changes in BCAA metabolism. This may be especially true if confined to only a subset of BCAA-consuming tissues. Additionally, changes in tissue protein turnover because of energy restriction or altered protein intake may mask any subtle dietary changes in BCAA catabolizing enzymes. Third, the twice-daily 10-g protein supplement regimen used here may not be a sufficient dose of BCAAs to alter BCAA-associated enzymes or other enzymes related to protein turnover. Evidence for this can be gleaned from studies exploring the bioactivities of Leu, i.e., an ability to stimulate mammalian target of rapamycin complex 1 (36). In clinical trials using older and sedentary adults, a meal threshold of ~2.2 g of Leu has been shown to activate a mammalian target of rapamycin complex 1 adequately enough to stimulate muscle anabolism (37). At the 10-g total protein doses used in our study, the whey protein isolate supplement contained ~2 g of total BCAA/dose, of which ~1 g was Leu. If BCAA-associated enzyme gene expression and activity stimulation require a similar meal threshold for Leu, then larger supplemental protein and BCAA doses may be required to observe relevant changes in enzymology and attendant fasting blood BCAA concentrations.

In contrast to other studies (38–40), but consistent with another recent report (41), we did not observe a decrease in plasma BCAA concentrations after weight loss. Laferre et al. (40) noted significantly greater reductions in BCAA concentrations after weight loss in individuals who have undergone gastric bypass surgery than in individuals with equal weight loss after dietary restriction. It is interesting to note that gastric bypass surgery dramatically reduces insulin resistance within 6 d after
surgery (42), and individuals undergoing gastric bypass surgery had significantly lower plasma glucose concentrations after an oral glucose tolerance test (40). Thus, greater reductions in BCAA concentrations observed in gastric bypass individuals may have been driven through improvements in insulin sensitivity rather than weight loss per se. Although both groups studied by Laferrère et al. (40) lost a considerable amount of total body weight and had improvements in indices of metabolic dysregulation (i.e., fasting glucose, HOMA-IR, leptin, and ghrelin), their mean postintervention BMIs were still higher than the mean baseline BMI in the current study. This suggests that not only is insulin resistance an important determinant of plasma BCAA concentrations, but the degree of insulin resistance coupled to adiposity is a key factor.

In the current study in obese women with metabolic syndrome, we observed a significant correlation between preintervention fasting plasma BCAA concentrations and HOMA-IR, a marker of insulin resistance. These findings are consistent with many other reports (1, 3, 6, 7, 43). We have interpreted these observations to mean that insulin resistance contributes to the BCAA phenotype and not the other way around. Despite strong evidence that diets rich in protein or dairy can have positive effects on metabolic health outcomes such as blood sugar control (9, 44), it has been asserted that more sulfur-containing amino acids in whey protein isolate than in gelatin (21). As a protein source, gelatin is severely deficient in sulfur-containing amino acids, with <1% of the total amino acids consisting of Met or Cys. Higher plasma cystine and cysteine in the gelatin-supplemented group although there is ~5 times more traditional approaches.

A novel result from this study was the identification of a set of plasma metabolites that accurately discriminate individuals consuming a relatively small amount of disparate protein sources, using an untargeted metabolomics analysis. Unlike most nutritional intervention studies that have used metabolomics for dietary biomarker identification (46–50), we specifically used fasting samples where the influence of acute postprandial supplement-derived metabolite excursions are negated. Under these conditions, interrogation of the plasma metabolome reveals metabolic pathways that are differentially reset by chronic exposure to a dietary protein shift. We observed higher plasma abundances of Cys- and Pro-related metabolites in participants consuming gelatin protein supplements, whereas plasma abundances of cholesterol, adenosine-5-phosphate, and glyceric acid were higher with dietary consumption of whey protein supplements. It is worth noting that the gelatin-based supplements contain 2.5 times more Pro than the whey-based supplements (21), which may account for the relatively higher abundance of 4-hydroxyproline, trans-4-hydroxyproline, and oxoproline in the gelatin-supplemented group. However, hydroxyprolines are generated through post-translational modification of Pro residues during protein synthesis and would not be isolated in plasma unless released during protein catabolism (i.e., from collagen breakdown or other proteins containing these residues) (51). Therefore, the appearance of these Pro derivatives must have been associated with an alteration of protein metabolism, likely through collagen catabolism, rather than the degradation of excess dietary Pro consumption. Whether the higher Pro load in the gelatin-based supplement compared with the whey-based supplement caused this change in protein catabolism is a question that will have to be answered with more targeted analyses. Overall, it highlights the efficacy of untargeted metabolomics as a tool to interrogate under-reported metabolic pathways that may not have been discovered using more traditional approaches.

Similar to Pro, plasma Cys and Cys derivatives were higher in the gelatin protein supplement group although there is ~5 times more sulfur-containing amino acids in whey protein isolate than in gelatin (21). As a protein source, gelatin is severely deficient in sulfur-containing amino acids, with <1% of the total amino acids, which may account for the relatively higher abundance of 4-hydroxyproline, trans-4-hydroxyproline, and oxoproline in the gelatin-supplemented group. However, hydroxyprolines are generated through post-translational modification of Pro residues during protein synthesis and would not be isolated in plasma unless released during protein catabolism (i.e., from collagen breakdown or other proteins containing these residues) (51). Therefore, the appearance of these Pro derivatives must have been associated with an alteration of protein metabolism, likely through collagen catabolism, rather than the degradation of excess dietary Pro consumption. Whether the higher Pro load in the gelatin-based supplement compared with the whey-based supplement caused this change in protein catabolism is a question that will have to be answered with more targeted analyses. Overall, it highlights the efficacy of untargeted metabolomics as a tool to interrogate under-reported metabolic pathways that may not have been discovered using more traditional approaches.

Interestingly, although we had 2 measurements of cholesterol, only the measurement from the global metabolomics profiling method was discriminant of protein supplementation. This example highlights the limitations with the global profiling method. Most notably, measurements from this method are semiquantitative and reflect ion abundances, which may not always correlate with targeted methods that provide absolute

![FIGURE 3](https://example.com/figure3.png)
concentration values. Also, the statistical analysis of high-dimensional data is subject to overfitting and overinterpretation because of the "small n/large p" paradigm. We used a training and test data splitting scheme to minimize these limitations, but we recognize that it is not a cure-all treatment. Therefore, we should clearly consider the modeling results as hypothesis generating until confirmed with more targeted approaches.

Our study has several limitations. Perhaps most notable is the likely variability among participants’ dietary intake outside of the protein supplementation in this study. Although instructional binders were provided that gave structured and homogeneous meal plans among all participants, their diets were likely highly variable at a given feeding. Thus, we cannot account for the type and total amount of consumed protein outside of the 20-g/d whey or gelatin supplementations. This study was a secondary analysis in a weight-loss study that was not powered to investigate changes in plasma BCAA abundance. The small sample size and slightly unbalanced treatment groups may have contributed to the observed differences among BCAAs and other metabolites at baseline and might have affected our ability to detect BCAA responses to dietary protein supplements. Additionally, dietary consumption before the start of the study was not controlled for and was likely heterogeneous. Furthermore, BCAA metabolism and other metabolic processes may be subject to variability at different obesity BMI classifications. Additionally, weight loss may have confounded or hindered any beneficial

**FIGURE 4** PLS-DA characterization (model 1) of postintervention clinical and metabolomic measurements in women with metabolic syndrome after an 8-wk weight-loss regimen including 20 g/d of gelatin (n = 7) or whey protein (n = 11) supplements. Clustering of participants consuming whey or gelatin protein supplementations along latent variables 1 and 2 of PLS-DA scores plot (A). Confidence regions of each cluster are presented as 95% confidence ellipses based on Hotelling’s $T^2$ statistic. Variables associated with each cluster (i.e., higher in individuals within a cluster) along latent variables 1 and 2 of PLS-DA loading plots (B). Variables in PLS-DA model had bootstrapped VIP distributions $\geq 1$. Only annotated variables are labeled for clarity. Unknown plasma metabolites are provided in Supplemental Table 2. Data for multivariate analyses were normalized, mean centered, and scaled to unit variance before analyses. HOMA, homeostasis model assessment; PLS-DA, partial least-squares-discriminant analysis; VIP, variable importance in projection.

**FIGURE 5** PLS-DA characterization (model 2) of postintervention clinical and metabolomic measurements in women with metabolic syndrome after an 8-wk weight-loss regimen including 20 g/d of gelatin (n = 7) or whey protein (n = 11) supplements. Clustering of participants consuming whey or gelatin protein supplementations along latent variables 1 and 2 of PLS-DA scores plot (A). Confidence regions of each cluster are presented as 95% confidence ellipses based on Hotelling’s $T^2$ statistic. Variables associated with each cluster (i.e., higher in individuals within a cluster) along latent variables 1 and 2 of PLS-DA loading plots (B). Variables in the PLS-DA model had a significant treatment effect ($P \leq 0.05$) in ANCOVA analyses. Data for multivariate analyses were normalized, mean centered, and scaled to unit variance before analyses. BB, BinBase identification numbers; PLS-DA, partial least-squares-discriminant analysis.
effects of increased dietary BCAA consumption. Finally, because we did not have a negative control group (e.g., without protein supplementation), we were unable to distinguish between weight loss–or supplementary protein–associated effects.

In conclusion, we found only modest evidence that a 10-g/dose twice-daily supplemental protein source comparatively rich in BCAs robustly modifies innate BCAA metabolism during an 8-wk period of moderate energy restriction in obese women with metabolic syndrome. Similar to findings from other groups, a positive correlation between BCAs and HOMA-IR before the dietary intervention provides further evidence that BCAA metabolism is altered in the insulin-resistant state. However, this relation was less robust after the intervention and further investigation is needed to distinguish whether this observation is independent of weight loss. Finally, using untargeted metabolomics, individuals consuming a whey-based protein supplement in addition to their calorie-restricted diet had lower fasting plasma abundance of Pro- and Cys-related metabolites than gelatin-based protein supplementation. The accurate discrimination of individuals consuming a low dose of protein from disparate sources provides evidence that even very small dietary protein modifications affect body-wide protein metabolism and that these changes can be detected using sensitive analytic metabolomics approaches. Our results suggest that whey and gelatin supplementation differentially affect Pro and Cys metabolism, and the nature of these relations warrants further investigation. Many discriminating metabolites are yet to be annotated and efforts to determine their chemical identities will provide new insights into metabolic changes stemming from whey consumption. The metabolomics results in this study should be validated in larger, broader populations, in response to varying doses of disparate dietary protein sources, and under conditions of weight maintenance.

Acknowledgments
We thank Rebekka Zak for helping in coordinating the study; Barbara Gale, for body composition measurements; and Rogelio Almario, for conducting laboratory assays. KBC, SEK, and SHA designed the research; KBC, SEK, and OF conducted the research; KBC, SEK, TAK, and OF provided essential reagents and materials; BDP analyzed data and wrote the manuscript; BDP and SHA interpreted the data and had primary responsibility for the final content and edits. All authors read and approved the final manuscript.

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