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Dietary Methionine and Total Sulfur Amino Acid Restriction in Healthy Adults

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Abstract

Objectives: Dietary restriction of methionine (Met) and cysteine (Cys) delays the aging process and aging-related diseases, improves glucose and fat metabolism and reduces oxidative stress in numerous laboratory animal models. Little is known regarding the effects of sulfur amino acid restriction in humans. Thus, our objectives were to determine the impact of feeding diets restricted in Met alone (MetR) or in both Met and Cys (total sulfur amino acids, SAAR) to healthy adults on relevant biomarkers of cardiometabolic disease risk.

Design: A controlled feeding study.

Setting and participants: We included 20 healthy adults (11 females/9 males) assigned to MetR or SAAR diet groups consisting of three 4-wk feeding periods: Control period; low level restriction period (70% MetR or 50% SAAR); and high level restriction period (90% MetR or 65% SAAR) separated by 3–4-wk washout periods.

Results: No adverse effects were associated with either diet and level of restriction and compliance was high in all subjects. SAAR was associated with significant reductions in body weight and plasma levels of total cholesterol, LDL, uric acid, leptin, and insulin, BUN, and IGF-1, and increases in body temperature and plasma FGF-21 after 4 weeks ($P<0.05$). Fewer changes

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occurred with MetR including significant reductions in BUN, uric acid and 8-isoprostane and an increase in FGF-21 after 4 weeks (P<0.05). In the 65% SAAR group, plasma Met and Cys levels were significantly reduced by 15% and 13% respectively (P<0.05).

Conclusion: These results suggest that many of the short-term beneficial effects of SAAR observed in animal models are translatable to humans and support further clinical development of this intervention.

Keywords

sulfur amino acids; methionine; cysteine; diet; sulfur amino acid restriction

Introduction

The biological aging process in humans is associated with a progressive deterioration of functional capacity and increases in the incidence of chronic diseases such as cancer, neurodegenerative diseases, and cardiovascular disease. Thus, a potentially effective approach to disease prevention is the development of strategies aimed at delaying or inhibiting specific aging processes that are linked to disease development. Previous preclinical studies have indicated that diets restricted in sulfur amino acids (SAA), methionine (Met) and cysteine (Cys), are effective at delaying the aging process and many of its associated detrimental changes when fed to laboratory animal models [1,2]. In 1993, it was first reported that lifelong feeding of an amino acid defined diet restricted in Met, as the sole source of sulfur amino acid, by 80%, resulted in >40% increases in median and maximum life span in rats [3]. While growth rate was reduced, young animals on the Met restricted diet appeared healthier than controls. Since these initial findings, similar life-span enhancing effects of Met restriction have been reported in numerous aging models including mice, Drosophila, yeast, and human cells in culture [4–8]. In addition to increasing life span, Met restriction has also been associated with improvements in a variety of aging-related impairments and diseases including reductions in insulin resistance, adiposity, oxidative stress, kidney disease and cancer [9–16]. Based on these results, Met restriction has become an important paradigm for delaying the biological aging process in laboratory animals [17].

While the mechanisms of Met restriction on aging have yet to be identified, low Met diets appears to improve overall metabolic health by impacting a number of critical metabolic and nutrient-sensing pathways [5,18]. Some of the most noted effects include improved glucose metabolism, reduced accumulation of hepatic triglycerides, and favorable changes in plasma biomarker levels including elevated adiponectin and FGF-21 levels and reduced leptin and IGF-1 levels [9,10,18]. Met restriction is also associated with overall reductions in oxidative stress and related biomarkers [14,19,20] and changes in a variety of critical cellular regulatory mechanisms including one-carbon metabolism [11], autophagy [21] and DNA methylation [22]. While much of the earlier literature has focused on the effects of Met restriction using defined amino acid diets devoid of Cys, results from later studies indicate that addition of Cys to the diet can eliminate many of the beneficial effects of Met restriction in rodents [22–26]. Overall, it has become apparent that reductions in both Met and Cys in the diet are required for maximum benefits. This is particularly relevant for translational consideration since Met and Cys are both common constituents in the diet.

Overall, the preclinical studies suggest that dietary total sulfur amino acid restriction is effective at delaying aging in animal models, however, there is little known regarding the translational significance of these findings in humans [2]. This could be of particular importance since most adults eat diets that are well in excess of their dietary requirement for SAA [27]. The majority of preclinical studies have been conducted in young and growing animals which have higher dietary SAA requirements than mature adults based on excess needs for growth [28]. Thus, SAA restricted diets initiated in young animals cause substantial reductions in growth. While translation of this dietary intervention in children may not be justified, the feasibility of this approach in adults is supported by findings in rodents showing that SAA restriction is effective even when initiated in adult animals including increasing longevity in mice [29], rats [30] and *Drosophila* [31] and restoring a more youthful phenotype in older mice [6,20,22]. Further, it has been noted that many vegan diets are naturally low in SAA due to the low Met and Cys content of many vegetable proteins [32]. Thus, while SAA restriction may represent a potentially feasible intervention in humans, there have been few controlled feeding studies reported to date. Here, we report on one such clinical study aimed at assessing the short-term effects of feeding diets restricted in Met alone (MetR) or in both Met and Cys (SAAR) to healthy adults on relevant biomarkers of cardiometabolic disease risk.

Materials and Methods

Study subjects

All participants provided written informed consent, and the Pennsylvania State University Institutional Review Board approved the protocol (STUDY00000302). Healthy subjects were recruited based on word of mouth, fliers and the ClnicalTrials.gov website (NCT02192437). While subjects were not blinded, study personnel involved in the analyses of samples and data were blinded to group assignments.

Inclusion criteria: Healthy English-speaking, males and females, 24–65 years of age, categorized as healthy weight to Class 1 obese (BMI=18.5–35 kg/m^2) based on CDC criteria (https://www.cdc.gov/obesity/adult/defining.html). Exclusion criteria: major medical condition or chronic disease including diabetes, PKU, mental illness, drug dependency, pregnant or nursing female, use of medications which could impact outcomes including anti-inflammatory drugs, corticosteroids, statins, and diabetes drugs, alcohol dependency, medications known to affect body weight, use of high dose dietary antioxidant supplements in past month, use of tobacco products in the past 6 months, unstable weight (loss or gain of >10%) over past 3 months, and allergies to eggs, wheat, nuts, soy and latex. Pregnancy tests were conducted at screening and at beginning of each diet period.

Study design

The design of this controlled feeding study consisted of two randomized groups as described in Figure 1A. After screening, eligible subjects were randomized to one of two diet groups as follows: In the MetR group, subjects were placed on a control diet (30.1 mg/kg/day Met; 30.1 mg/kg/day Cys) for 4 weeks followed by a 3–4 week washout period, then a 70% MetR diet (9.0 mg/kg/day Met; 30.1 mg/kg/day Cys) for 4 weeks, followed by another 3–4 week

washout period and finally a 90% MetR diet (3.0 mg/kg/day Met; 30.1 mg/kg/day Cys) for 4 weeks. In the SAAR group, subjects were placed on a control diet (30.1 mg/kg/day Met; 30.1 mg/kg/day Cys) for 4 weeks followed by a 3–4 week washout period, then a 50% SAAR diet (15.0 mg/kg/day Met; 15.0 mg/kg/day Cys), followed by another 3–4 week washout period, and finally a 65% SAAR diet (10.4 mg/kg/day Met; 10.4 mg/kg/day Cys) for 4 weeks. A flow chart of participant enrollment and participation prepared according to CONSORT standards is provided in Figure 1B. The were no available data on specific effects of MetR or SAAR on the proposed outcomes at the time of designing this study, so power calculations were based on normal levels from previous clinical studies and expected changes based on laboratory animal studies. For plasma methionine, using baseline data from healthy controls [33] and changes resulting from SAAR diets in rats [34], a sample size of 10 was expected to allow for 95% power (α=0.05) to observe significant differences (62%) pre and post feeding.

Potential subjects were initially screened by telephone to obtain information on demographics, occupation, lifestyle habits, and other eligibility criteria. Eligible subjects were invited to an in-person screening where informed consent was obtained and body mass index (BMI), medical history and a blood sample was obtained. Potential participants met with the study nutritionist to complete a physical activity recall and assessment for potential taste aversion to study foods.

Prior to each diet period, subjects completed three random, unannounced 24-hour telephone dietary recalls conducted by the study nutritionist to evaluate usual diet practices. Dietary intake data were collected and analyzed using Nutrition Data System for Research software version 2011 developed by the Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN.

Study diets

During the test diet periods, all foods consumed were prepared at the Penn State Clinical Research Center (CRC) Metabolic Kitchen under the direction of a trained nutritionist. Subjects visited the CRC each weekday and were weighed and provided their food for that day, eating one meal in the CRC dining room. Also, at each visit, subjects completed a daily diet monitoring questionnaire to record any deviations from dietary protocols. On Fridays, subjects were provided with their food for the weekend. Subjects were instructed to eat only and all of the food provided.

All study diets were designed to provide 50–53% of calories as carbohydrate, 35–38% as fat and 12–13% as protein/amino acids. For each diet, 20–25% of proteins were from low SAA sources including fruits, vegetables and refined grains. The remaining 75–80% were obtained from protein/amino acid dietary supplements as follows: Control diet - mixture of sesame flour (Sesmena, Depasa, USA, Brownsville, TX) and egg white powder (GNC Optimum Nutrition. Pittsburgh, PA); MetR diet – Met-free amino acid-based medicinal drink (X-Met Maxamaid, Nutricia, Schiphol, The Netherlands); SAAR diet - Cys- and Met-free amino acid-based medicinal drink (X-Met X-Cys Maxamaid, Nutricia, Schiphol, The Netherlands). The nutrient content of all foods was calculated using Nutrition Data System for Research software version 2011 (NCC, University of Minnesota, Minneapolis,

MN) [35] Diet compositions were based on a 2500 kcal/day diet with individualized energy intake based upon study subject requirements (ranged from 1700 to 3800 kcal/day). During the first 2 weeks of the control feeding period, if changes in body weights were observed, dietary energy intake was adjusted so that body weight would be maintained. A six-day cycling menu schedule was used so that the same foods would not be provided the same day of the week (Sample 6-day menu for 70% MetR diet provided in Supplemental Table 1). The levels of Met (31.0 mg/kg/day) and Cys (31.0 mg/kg/day) in the control diet were selected based on reported estimates for total SAA intake from a typical balanced American diet (61 mg/kg/day for a 70 kg adult) [32] and the assumption that diets contain roughly similar levels of Met and Cys.

Chemical analysis of foods

To confirm the Met and Cys content of the diets, a sample of each day's diet, including supplemental amino acid mixtures for each diet group, based on 2500 kcal, was collected, homogenized in a food-grade blender and stored at −20°C for analysis of amino acid content. Based on the 6-day diet menu and 5 different diets (control, 70% MetR, 90% MetR, 50% SAAR and 65% SAAR), a total of 30 different samples were analyzed. Chemical analyses included calories, macronutrients, fatty acids and amino acids (Covance, Madison WI).

Primary, Secondary and other outcome measures

The primary outcome measures included body weight and plasma sulfur amino acids at baseline and four weeks for each diet period. The secondary outcome measures included biomarkers of oxidative stress and blood lipids at baseline and 4 weeks for each diet period. Several other outcome measures are listed in sections below and were also performed at baseline and 4 weeks for each diet period.

Collection of biological samples

On the first and last day of each diet period, fasting blood (~50 mL), and spot urine samples were collected and body weight, waist circumference and temperature were measured. Additionally, one week into the 2nd and 3rd diet period, blood samples were collected. Aliquot of whole blood was removed and analyzed immediately for creatine kinase to test for possible protein malnutrition. From the remaining blood, samples or whole blood and plasma as well as urine samples were frozen and stored (−80°C) until analysis.

Clinical chemistry

Clinical chemistry analyses were conducted at the Hershey Medical Center Clinical Laboratory. Plasma amino acid profiles were conducted at the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories (Columbia, MO). Blood glutathione, glutathione disulfide, glutathionylated proteins, and hemoglobin and plasma total cystine were analyzed as we described previously [36]. ELISAs were used for the measurement of urinary 8-Isoprostane (Cayman Chemical, Ann Arbor, MI) and plasma IGF-1, leptin, FGF-21, adiponectin, and CRP (R & D Systems, Inc., Minneapolis, MN).

Statistical methods

We used SAS software version 9.4 (SAS Institute, Inc., Cary, NC, USA) for all statistical analyses. Biomarker concentrations were modeled using log-transformed values for continuous variables. The continuous variables followed a Gaussian distribution. Group differences of baseline characteristics and biomarkers, as well as dietary nutrient intake, were compared by using analysis of variance (ANOVA) for continuous variables and chi-square for categorical variables. The primary comparisons of the study involved changes in outcomes observed during each study period. Secondary analyses involved comparison of changes in outcomes observed in the MetR-group control period versus the changes observed during the 70% MetR, 90% MetR study periods and changes in outcomes observed in the SAAR-group control period versus the changes observed during the 50% SAAR, 65% SAAR study periods. All reported P values are nominal two-sided P values, and significance was set at $P<0.05$. To further test for the robustness of the findings, we compared group differences over different sexes in the subgroup analyses. Significance was reported with Bonferroni adjustments with correction factor of (0.05/6 = 0.0083). The effect size between control and diet periods was determined by Cohen's *d* value (M1 - M2 / SD_{pooled}, where $SD_{pooled} = \sqrt{[(SD1^2 + SD2^2) / 2]}$).

Results

Study subject characteristics and baseline values

The study design included 2 diet groups (MetR and SAAR), n=10 per group (Figure 1A). Subjects did not differ in their age, gender, weight or BMI at baseline (Supplemental Table 2). The CONSORT flowchart of study participants is provided in Figure 1B.

Adverse effects and safety

No serious adverse effects were reported. Other adverse events, including either mild or moderate symptoms of colds, body pains, and headaches, were observed in 10% and 20% of subjects in MetR and SAAR groups, respectively. All blood markers of liver and kidney function or malnutrition were within normal range.

Confirmation of dietary intake interventions

A series of confirmatory steps, which included assessment of actual dietary intake on an individual basis and measured levels of Met and Cys in study diets, were conducted. Dietary analyses included that 5 different diet groups (Control, 70% MetR, 90% MetR, 50% SAAR and 65% SAAR) (Figure 1A). All diets provided nutritionally adequate levels of SAA according to Recommended Dietary Allowance (RDA) (19 mg/kg/day) and Estimated Average Requirement (EAR) (15 mg/kg/day) values for total SAA (Supplemental Figure 1) [37]. Diets were designed based on an energy intake of 2500 kcal/day and a body weight of 70 kg, with caloric intake individualized based upon energy requirements. When mean SAA intakes were calculated based on individual dietary intake and body weights, Met and Cys for each group were slightly higher than the dietary goals (Supplemental Figure 2).

Food samples from each of the 30 different diet group/days were analyzed for nutrient content (Supplemental Table 3) and measured SAA levels were compared with predicted

values (Supplemental Figure 3A). Met and Cys for each group were highly correlated with predicted values (r=0.98 for Met and r=0.96 for Cys) and only minor differences were observed for absolute values (mean percent differences for Met:10.6% and Cys:−9.1%). Using these values together with individual food intake and body weight data, actual SAA intake levels were highly comparable to SAA intake goals with only minimal differences noted (Supplemental Figure 3B).

Usual Dietary Intake Prior to Dietary Interventions

The SAA levels in the control diet was designed to deliver 60.2 mg/kg/day and was confirmed by chemical analysis of the foods consumed to be 68 mg/kg/day (approximately equally divided between Met and Cys) (Supplemental Figure 3). This design was based on the reported SAA intake from an average balanced American diet of 4.3 g/day [32] which equates to 61 mg/kg/day based on a body weight of 70 kg. Analysis of the usual diets during the week prior to each of the study feeding periods by 24-hr recall indicated that Met and Cys intake levels were similar prior to each of the experimental feeding period (Supplemental Figure 4) with a mean of 24.5 mg/kg/day for Met, 16.3 mg/kg/day for Cys and 40.8 mg/kg/day for total SAA. Thus, based on these usual intake values, switching to the control diet was associated with an increase in total SAA intake of ~40%. This increase in SAA could have potentially impacted some of the outcomes such as the increase in urinary 8-isoprostane observed during the control diet period. Further, since the starting levels of SAA intake were low in each of the feeding periods compared to control, the actual relative SAA reductions were substantially less than anticipated for MetR (62% and 87% for the 70% MetR and 90% MetR groups, respectively) and for SAAR (23% and 47% for the 50% SAAR and 65% SAAR groups, respectively).

Biomarkers Analyses

At baseline for each of the three feeding periods for both MetR and SAAR groups, there were no significant differences in anthropometric measures, metabolic markers or risk factors (Supplemental Tables 5 and 6). Changes from baseline in anthropometric measures, cardiometabolic disease markers and risk factors in subjects from the MetR group are summarized in Figure 2 as well as in Supplemental Table 7 and from the SAAR group are summarized in Figure 3 as well as in Supplemental Table 8. SAAR was associated with significant reductions in body weight and plasma levels of total cholesterol, LDL, uric acid, leptin, and insulin, BUN, and IGF-1, and increases in body temperature and plasma FGF-21 after 4 weeks ($P<0.05$). Fewer changes occurred with MetR including significant reductions in BUN, uric acid and 8-isoprostane and an increase in FGF-21 after 4 weeks ($P<0.05$). Changes in additional disease risk indices for cardiovascular disease, diabetes and coronary artery disease measured by change in waist: height ratios, HOMA-IR and remnant cholesterol showed a nonsignificant decreasing trend for both MetR and SAAR groups (Supplemental Table 9).

For those biomarkers which were significantly impacted by dietary SAA restriction, the effects of both MetR and SAAR diets are compared in Figure 4. Effects of SAAR on body weight (control vs. 50% restriction; $d = 1.01$, control vs. 65% restriction; $d = 1.96$), waist circumference (65% restriction), temperature (control vs. 65% restriction; $d = -0.99$), BUN

(65% restriction), LDL (control vs. 50% restriction; $d = 0.88$, control vs 65% restriction; $d = 0.43$), insulin (control vs. 50% restriction; $d = 0.91$, control vs 65% restriction; $d = 0.46$), IGF-1 (65% restriction), FGF-21 (65% restriction), and leptin (control vs. 50% restriction; $d = 0.82$, control vs 65% restriction; $d = 1.0$) were significantly different from changes in the corresponding MetR feeding period. Biomarker differences by sex for the MetR and SAAR groups are provided in Supplemental Figures 5 and 6, respectively. For both MetR and SAAR groups, similar trends were observed for both males and females, although fewer statistically significant changes were observed overall due to the reduced sample size.

Amino acid levels were analyzed in plasma from MetR and SAAR participants and changes are summarized in Supplemental Tables 10 and 11, respectively. Plasma levels of Met and total Cys trended downward with both MetR and SAAR, however, the only significant decreases occurred with 65% SAAR group when plasma Met and Cys levels were significantly reduced by 15% and 13% respectively (Figures 5A and 5B). Significant reductions in all three branched chain amino acids (BCAA) leucine, isoleucine and valine, were observed with both 50% and 65% SAAR, while with 90% MetR, significant decreases were only observed for valine and leucine (Figure 5C, left panel). Intake of branched chain amino acids, calculated based upon individual food intake and measured amino acid levels in foods, did not differ across all diet periods (Figure 5C, right panel). Plasma proline levels were decreased significantly only during the control period for the MetR group and during all feeding periods for the SAAR group despite proline intake being 40–50% higher in both MetR and SAAR feeding periods (Figure 5D). Plasma tyrosine levels were decreased significantly by in the 50% and 65% SAAR groups while intake levels did not differ across all the diet periods (Figure 5E).

Effects of MetR and SAAR feeding on other plasma clinical chemistry measures including electrolytes, liver enzymes, bilirubin and iron are provided in Supplemental Tables 12 and 13, respectively. No significant changes were observed during any of the diet periods with the exception of alanine aminotransferase (ALT) which was significantly decreased (16%) during the control period in the MetR group.

Discussion

Feasibility of total sulfur amino acids restriction in healthy adults

Results from the SAAR group in this controlled feeding study indicate that sulfur amino acid restriction without calorie restriction is a feasible, safe and effective intervention for reducing body weight, waist circumference and for inducing beneficial changes in biomarkers of cardiometabolic disease risk. Further, these beneficial changes occurred in as little as 4 weeks after initiation of the restricted diets and occurred similarly in both males and females. For most biomarkers, changes were in the direction of a reduction in risk, with the exception of adiponectin, which was slightly decreased in 65% SAAR period. In contrast to the SAAR, MetR resulted in decreases in uric acid, BUN, and 8-isoprostane levels and an increase in FGF-21 levels suggesting that reductions in both Met and Cys intake are required for many of the other changes resulting from SAAR. Interestingly, in addition to plasma SAA, reductions in BCAAs, were also observed with decreasing SAA intake and some of the effects of SAAR observed in the present study, may be attributed to reductions in

BCAAs. No major changes were observed for levels of total protein, albumin, and creatine kinase suggesting that the MetR and SAAR diets were safe and not associated with muscle wasting or any other negative health effects.

Similarity among changes in humans and animals following SAAR diet

Overall, the changes associated with the SAAR diet were consistent with those previously observed for similar diets in laboratory animals. SAA restriction in a variety of animal models was associated with decreases in body weight, uric acid, BUN, LDL, IGF-1, FGF-21, leptin, insulin and 8-isoprostane [1–3,5,10,14]. It is important to note that most of the previous Met restriction studies in laboratory animals used Cys-free defined amino acid diets and, as such, the experimental diets were restricted in both Met and Cys. The fact that similar changes were observed for these important biomarkers in the current randomized controlled feeding study suggest that SAAR is inducing similar beneficial changes on metabolism and disease processes as observed in preclinical models. However, a robust increase in adiponectin and reduction in glucose observed in rats and mice were not observed by SAAR in the present study. It is of interest that FGF-21 increases were actually greater for MetR than for SAAR. FGF-21 is thought to play an important role in glucose homeostasis and be a key contributor to the methionine restriction-dependent life span extension [38]. Circulating and hepatic FGF-21 levels are consistently elevated in MetR rodents and enhances glucose and lipid metabolism [1]. However, other studies in obese mice demonstrate a lack of dependency of both FGF-21 and adiponectin on weight loss and body composition [39]. Metabolically healthy human subjects undergoing short-term protein restriction exhibit elevated circulating FGF-21 concentrations [40–42]. While, a high protein diet by diabetic patients with non-alcoholic fatty liver disease show reduced FGF-21 concentrations [43,44]. However, as noted above, FGF-21 was enhanced in both diets and, thus, cannot explain observed differences in other endpoints. Previous animal studies suggest that SAAR is associated with reductions in oxidative stress [14,19]. In the rat, decrease in 8-isoprostane was observed as soon as 4 weeks after initiation of the Met restricted diet [14]. In the present study no changes from baseline were observed for urinary 8-isoprostane levels in either MetR or SAAR groups. However, during the control period, large increases (52–119%) in 8-isoprostane levels were observed. Thus, it appears that the control diet has pro-oxidant effects which are mitigated by both MetR and SAAR.

Comparative SAAR interventions in humans

To our knowledge, this is the first randomized human controlled feeding study examining both MetR and SAAR in healthy adults. In a recent one-week pilot study involving overweight or obese women which included 3 different SAA diets, 2 of which were similar in SAA intake to our control and 65% SAAR diets, results indicated that dietary Met and Cys restriction increased circulating levels of FGF-21, and had a beneficial impact on subcutaneous adipose tissue gene expression [45]. In a previous clinical trial in individuals with metabolic syndrome, subjects were instructed to abstain from dietary sources of protein and to replace their protein/amino acid requirement with a Met-free amino acid-based medical food, Hominex-2 [46]. However, while the target Met intake level in the MetR group (2 mg/kg/day) of that previous study was similar to the Met goals in the 90% MetR group in the present study, Cys was provided in fully adequate

amounts; thus, the impact of SAAR was not addressed. A similar MetR diet was also tested in our previous controlled feeding study in healthy adults [11]. While this diet produced plasma metabolomic profiles similar to those observed in Met restricted mice, the effects of SAAR were also not addressed. To provide specific information on the impact of both MetR and SAAR, in the present study we selected to use a controlled feeding design where each of the participants' diets were individually personalized based upon their metabolic requirements and all foods were produced and provided through the study's metabolic kitchen. Further, the actual SAA intake levels were confirmed through the analysis of study diets, the results of which provided a high degree of concordance with target SAA intake goals. Altogether, these findings confirm the robustness of the study intervention, and strengthen the conclusions which can be drawn regarding the impact of MetR and SAAR. It is also important to note that the close concordance of actual content of Met and Cys in study foods as assessed by direct measurement with predicted levels based on the use of the USDA Food Composition Database ($r=0.96$ for Met and $r=0.98$ for Cys) provides strong support regarding the accuracy of that database for SAA for future studies involving diet development or nutrient intake assessment.

Our present results are consistent with our recent nutritional epidemiologic studies of usual SAA intake and diabetes mortality and biomarkers of cardiometabolic disease risk factors in nationally representative cohorts of CVD-free adults [27,47]. Furthermore, in this cross-sectional study, higher intake of SAA was associated with increased levels of cardiometabolic disease risk biomarkers including several that were found to be impacted in the present controlled feeding study (cholesterol, uric acid, BUN and insulin). Interestingly the SAA intake levels in the lowest SAA intake quintile group ranged between 15–24 mg/kg/day, levels similar to the present 65% SAAR group (20.8 mg/kg/day). This emphasizes the relevance of the diet groups used in the present study and supports the feasibility of SAAR in the general and disease population.

Despite the large decreases in SAA intake in the individuals in both MetR and SAAR groups, only modest (~15%) decreases in plasma Met and total Cys levels were detected, and those were primarily observed in the most severe SAAR group (Figures 5A and 5B). These findings are consistent with previous studies where the degree of Met or Cys reduction in plasma after feeding MetR or SAAR diets was substantially lower than the reduction in levels of intake in both laboratory animals [10,23,48] and in clinical studies [46,49]. In our study, the decreases in Met and Cys in plasma were minor for the MetR group, most likely because of the ample Cys levels provided in that diet formulation. Indeed, levels of total SAA intake reduction for 70% MetR and 90% MetR groups were only 35% and 45%, respectively. Higher plasma SAAs levels have been strongly and independently associated with incident diabetes [50], further supporting the importance for the need for reducing intake of total SAA for maximum effectiveness, and to potentially, prevent or slow down chronic diseases like diabetes.

Comparing branched chain amino acids with total sulfur amino acids

Interestingly, in addition to plasma SAA, reductions in BCAAs were observed with decreasing SAA intake (Figure 5C). These reductions were not due to changes in BCAA

intake, as intake levels were constant across all diet groups. The reduction in BCAA levels may be due, in part, to the lack of animal-derived proteins in the test diets. In a previous clinical feeding study, decreases in both SAA and BCAA levels in plasma were shown to result in individuals transitioning to a vegan-based diet enriched with fish [51] and meat-lovers versus vegans [52]. Higher circulating BCAA levels have been associated with a variety of cardiometabolic risk factors including obesity, insulin resistance, and dyslipidemia [53,54]. Likewise, higher circulating levels of Cys have been linked to obesity, adiposity and insulin resistance [55]. Circulating SAA and BCAA levels may be similarly impacted by diet as both are associated with diets low in meat and high in vegetable proteins [27,52]. In a study where participants were switched to a vegan diet, both circulating BCAA and SAA were reduced [56]. Overall, the possibility that some of the effects of SAAR observed in the present study, may be attributed to reductions in BCAAs is worthy of consideration, as is further examination into the relationships between SAA and BCAA status.

Impact of SAAR on other amino acids

SAAR resulted in reductions in plasma proline and tyrosine, neither of which could be explained by reductions in dietary intake of these amino acids. Also, like BCAAs and SAA, circuiting levels of proline and tyrosine were decreased in adults when switching to a vegan diet [56]. Plasma tyrosine levels were also significantly reduced in individuals who reported usually eating primarily a vegan diet [52]. Significant decreases in both 1-methylhistidine and 3-methylhistidine were observed for all diet periods, including control, for both MetR and SAAR groups. This is likely due to the lack of meat in the test diets as both 1-methylhistidine and 3-methylhistidine levels in plasma have been shown to be sensitive indicators of dietary meat intake [57].

SAA intake levels crucial for better outcomes: lessons learned

In this study, the most effective results were observed with the most severe SAAR diet (65% SAAR). This diet was confirmed to provide 25 mg/kg/day SAA, slightly over the RDA for SAA of 19 mg/kg/day. It is not known if additional reductions in SAA to levels at or below the RDA or EAR would result in additional benefits or if differing ratios of Met:Cys may be more or less effective. In studies of adult initiation of Met restriction in rats, the dietary levels of SAA in the restricted groups (0.17%) [3,9,58] were slightly below the reported dietary requirements for mature rats (0.23%) [28]. Future studies will be necessary to determine the optimal dietary intake of SAA without negatively impacting protein synthesis, nitrogen balance or other endpoints related to Met as an essential nutrient.

It was of interest to note that in the most severe SAAR group (65%), a small (1%), but significant increase in body temperature was observed. In a previous study of SAAR restriction in rats, 1–2% increases in core body temperature were reported in adult animals, a thermogenic response related to an increase in total energy expenditure and uncoupling of respiration thought to be involved in the mechanisms of MetR through engagement of nutrient sensing pathways [59].

Limitations of the study

A limitation of the study design was the wide age range and BMI range of study subjects even though the subjects were randomized and their baseline BMI were not significantly different. Another possible limitation of the study design which included feeding periods with increasing levels of restriction (escalating dose design) without a cross-over as well as the potential for carryover from one feeding period to the next despite attempts to limit such effects through inclusion of a washout period. However, significant carryover is unlikely as no differences in study endpoints were observed between baseline values for each of the 3 feeding periods for both MetR and SAAR groups (Supplemental Tables 5 and 6).

Overall, these results demonstrate a number of beneficial effects of switching to a SAAR diet on a variety of anthropometric and cardiometabolic disease risk factors in healthy adults, similar to results observed in animal models. Hence, these findings suggest that many of the short-term effects of SAA restriction observed in animal models are translatable to humans and provide support for the further clinical development of this dietary intervention as a potential strategy for health promotion and disease prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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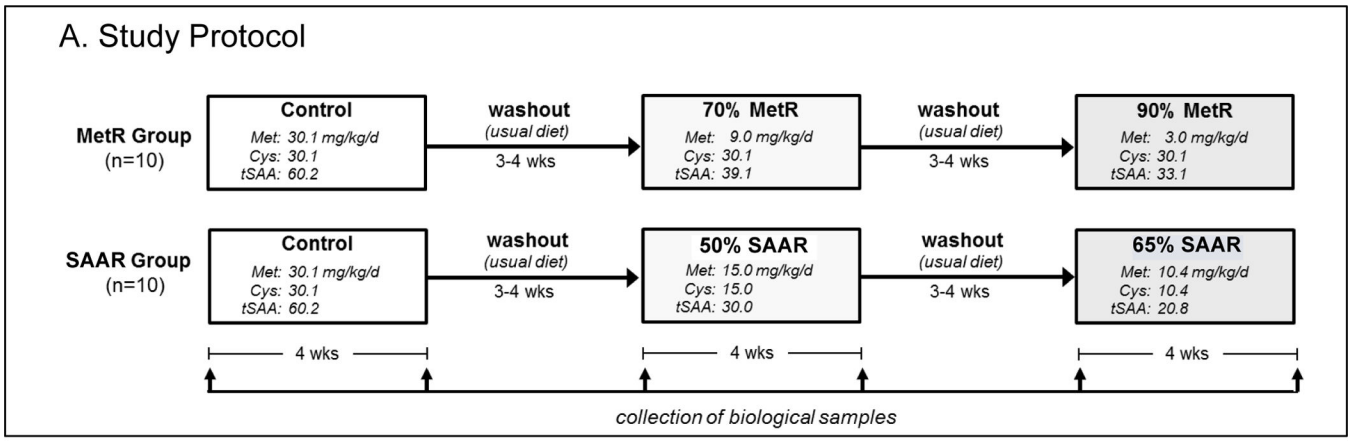

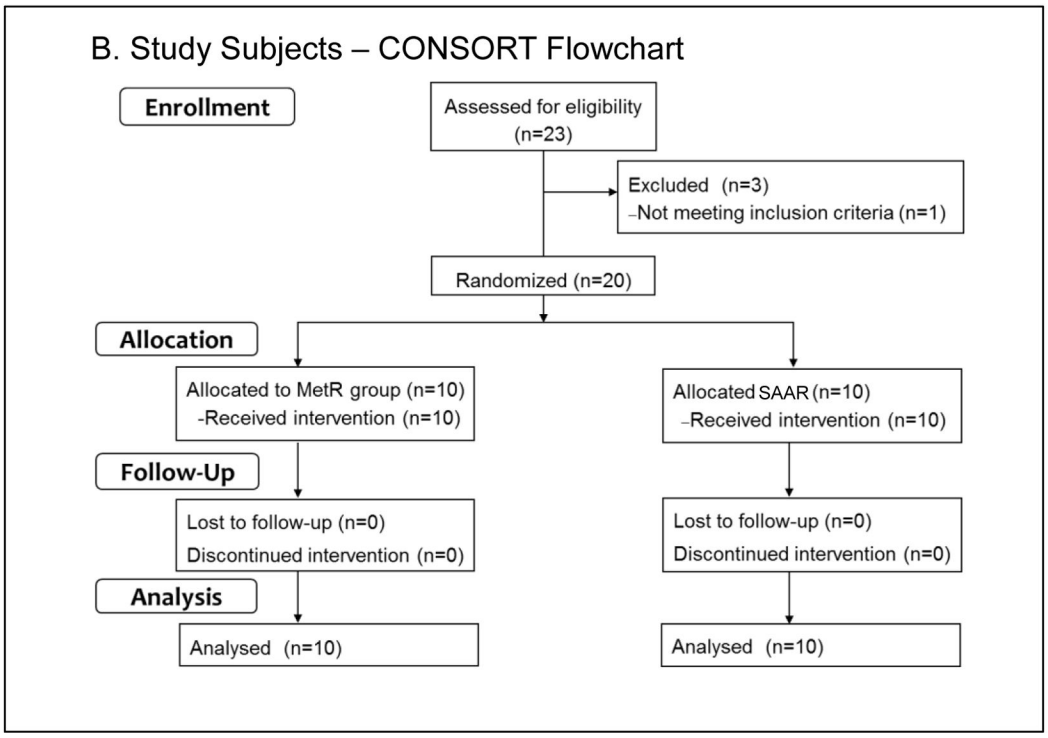

Figure 1.

Study Protocol and CONSORT Flowchart. A. Diagram depicting study groups, diets and timeline. B. Consolidated Standards of Reporting Trials (CONSORT) diagram of 23 subjects contacted subjects, 20 of which were enrolled into two arms as follows: MetR arm (n = 10) and SAAR arm (n=10).

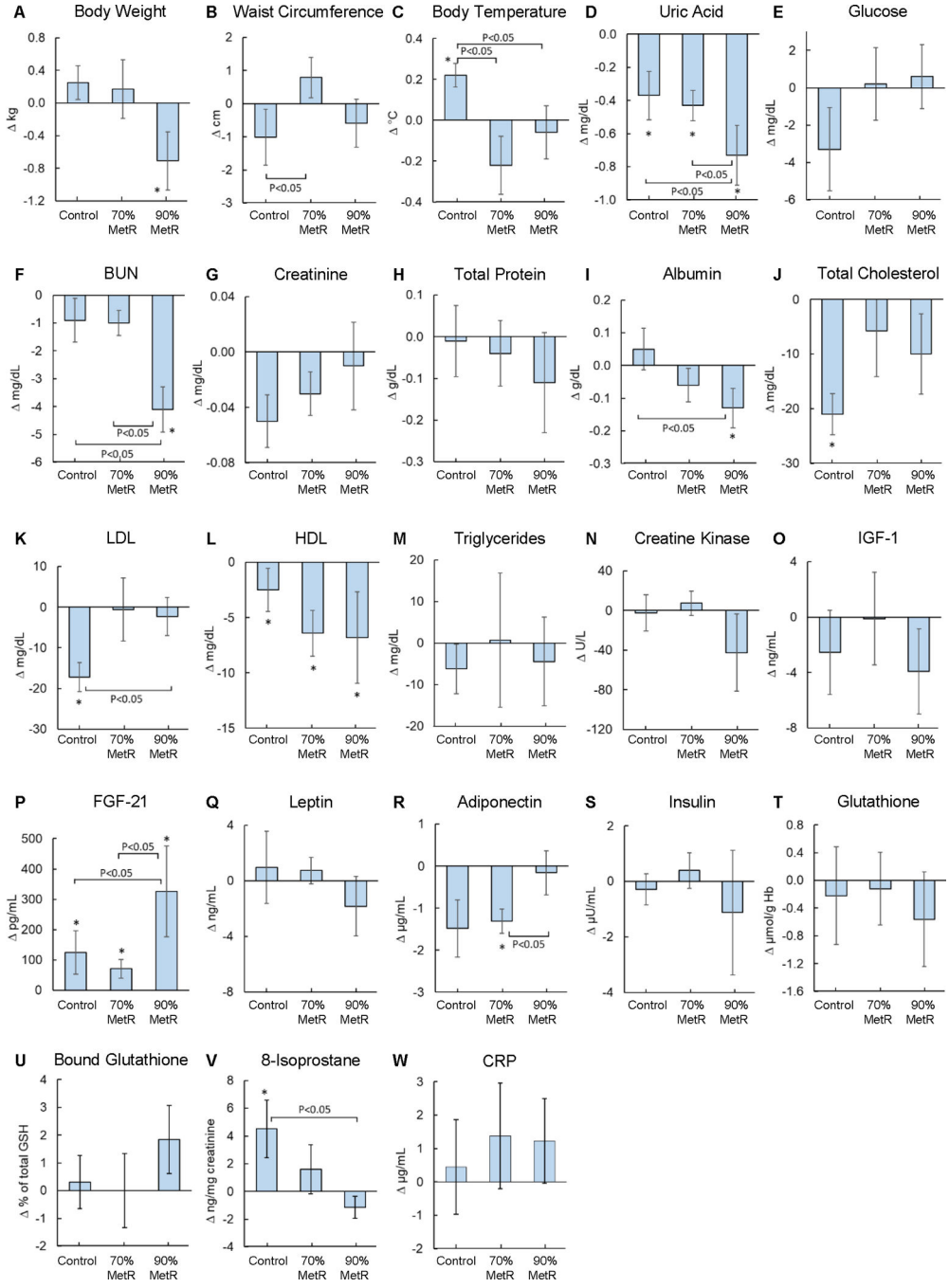

Figure 2.

Cardiometabolic disease biomarker/risk factor changes during control and MetR feeding periods. Within Group & Between Diet Period Comparisons. For subjects in the MetR group, changes from baseline after 4 weeks each diet period (control, 70% MetR and 90% MetR) are presented for body weight (A), waist circumference (B), body temperature (C), plasma uric acid (D), plasma glucose (E), BUN (F), plasma creatinine (G), plasma total protein (H), plasma albumin (I), plasma total cholesterol (J), plasma LDL cholesterol (K), plasma HDL cholesterol (L), plasma triglycerides (M), plasma creatinine kinase (N),

plasma IGF-1 (O), plasma FGF-21 (P), plasma leptin (Q), plasma adiponectin (R), plasma insulin (S), whole blood glutathione (T), whole blood protein bound glutathione (U), urinary 8-isoprostane (V) and plasma CRP (W). Bars are mean ± SE. *Statistically different change from baseline, ($P<0.05$).

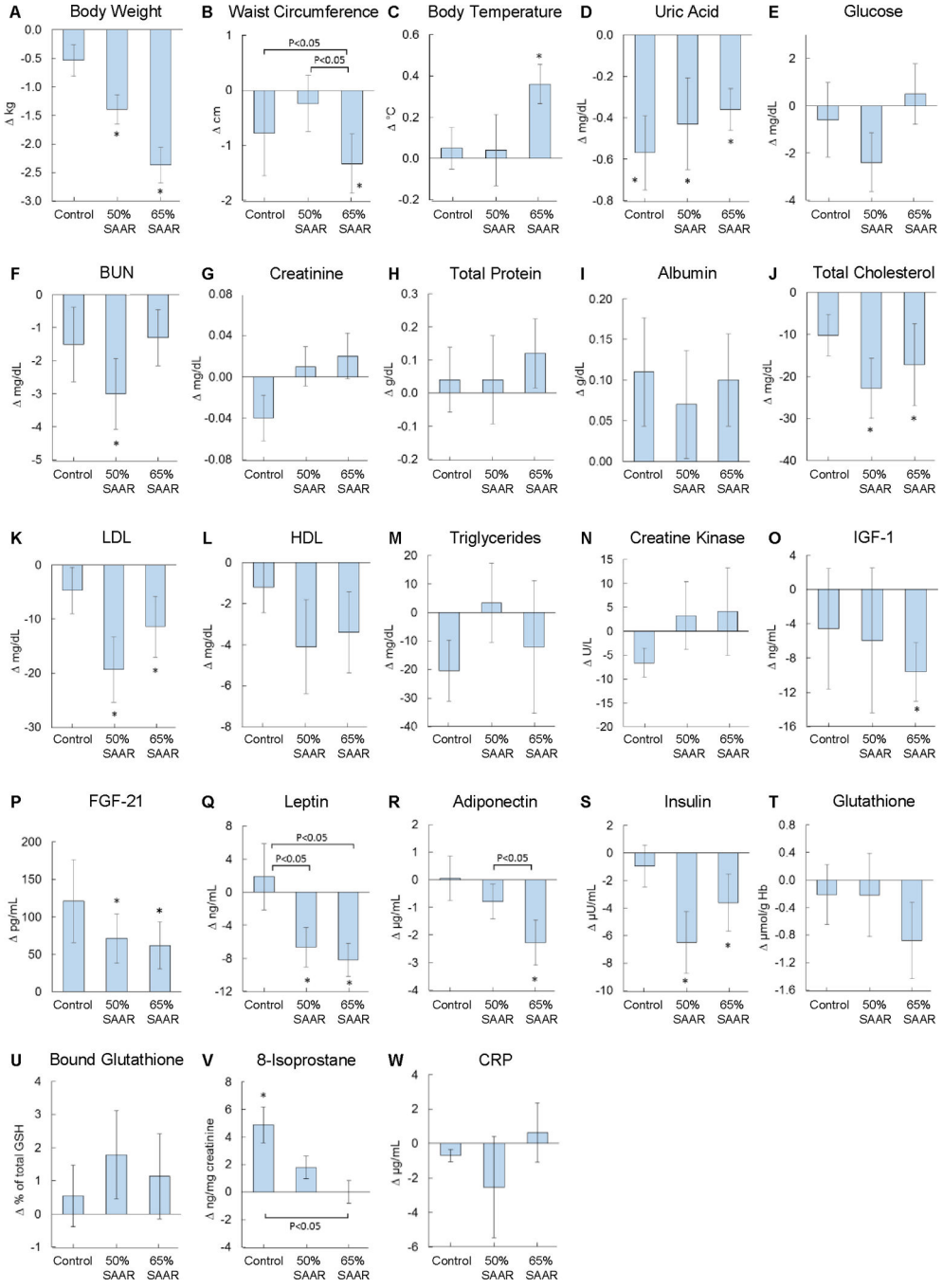

Figure 3.

Cardiometabolic disease biomarker/risk factor changes during control and SAAR feeding periods. Within Group & Between Diet Period Comparisons. For subjects in the SAAR group, changes from baseline after 4 weeks each diet period (control, 50% SAAR and 65% SAAR) are presented for body weight (A), waist circumference (B), body temperature (C), plasma uric acid (D), plasma glucose (E), BUN (F), plasma creatinine (G), plasma total protein (H), plasma albumin (I), plasma total cholesterol (J), plasma LDL cholesterol (K), plasma HDL cholesterol (L), plasma triglycerides (M), plasma creatinine kinase (N),

plasma IGF-1 (O), plasma FGF-21 (P), plasma leptin (Q), plasma adiponectin (R), plasma insulin (S), whole blood glutathione (T), whole blood protein bound glutathione (U), urinary 8-isoprostane (V) and plasma CRP (W). Bars are mean ± SE. *Statistically different change from baseline, ($P<0.05$).

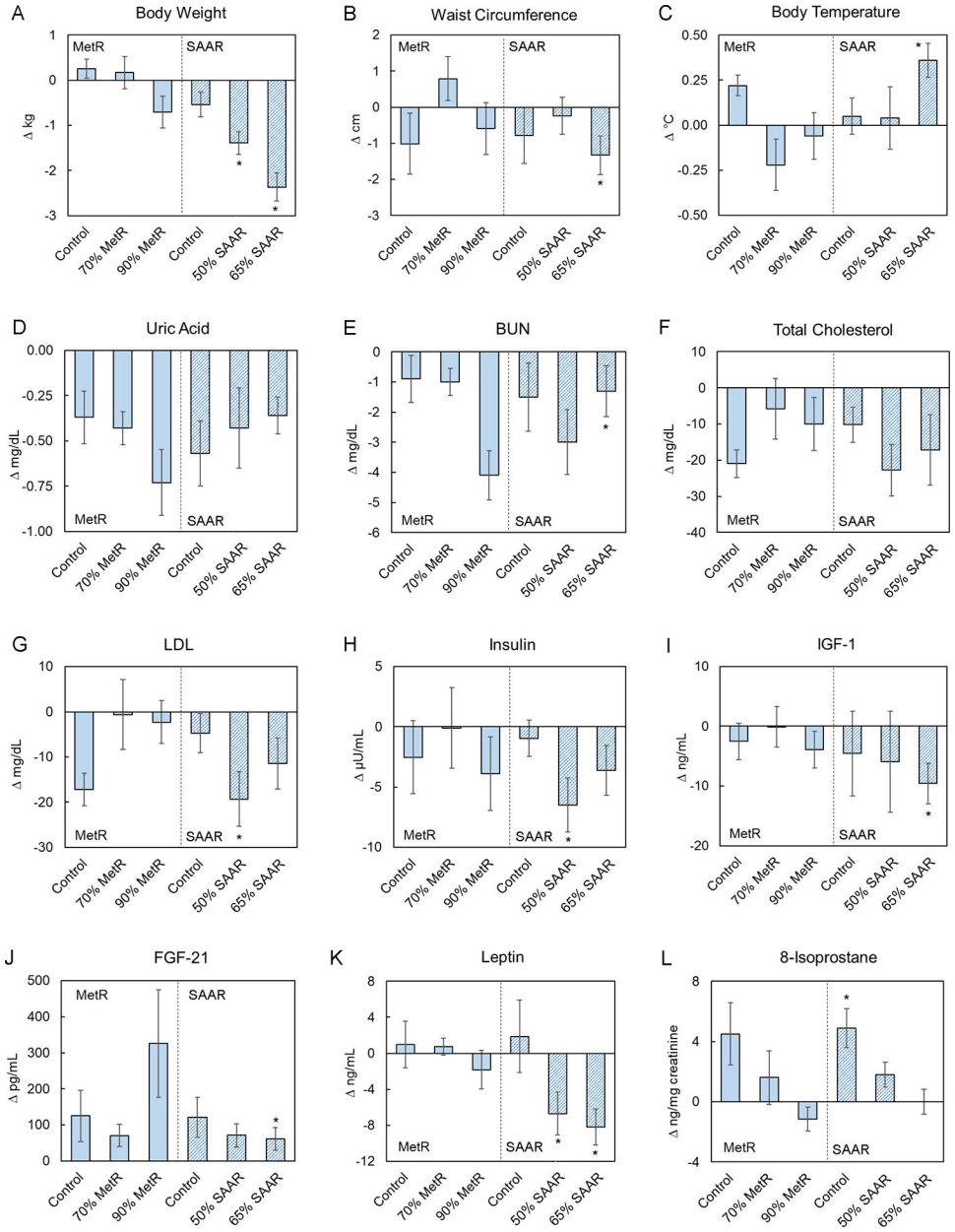

Figure 4.

Comparison of the Effects of MetR and SAAR Diets on Selected Biomarkers. Bars are mean ± SE. *Change from baseline significantly different from MetR group (P<0.05).

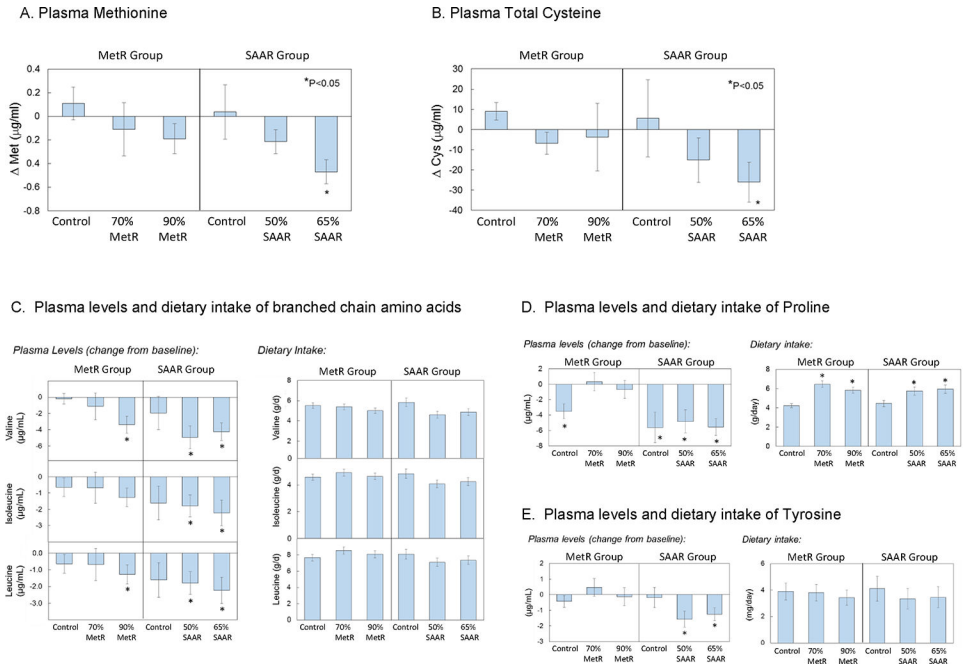

Figure 5.

Changes in Plasma Levels and Intake of Selected Amino Acids by MetR and SAAR. Plasma samples were obtained for all individuals before and after each diet period and analyzed for individual amino acid content. A. Changes in plasma Met levels during each diet period in both MetR and SAAR groups. B. Changes in plasma total cysteine (total reduced and oxidized forms) levels during each diet period in both MetR and SAAR groups. C. Changes in plasma branched chain amino acids (valine, isoleucine and leucine) levels during each diet period in both MetR and SAAR groups (left panel) and dietary intake of branched chain amino acids during each diet period in both MetR and SAAR groups (right panel). D. Changes in plasma proline levels during each diet period in both MetR and SAAR groups (left panel) and dietary intake of proline during each diet period in both MetR and SAAR groups (right panel). E. Changes in plasma tyrosine levels during each diet period in both MetR and SAAR groups (left panel) and dietary intake of tyrosine during each diet period in both MetR and SAAR groups (right panel). Bars are mean ± SE. *Statistically different change from baseline, ($P<0.05$).