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Metabolite Profiling Identifies Pathways Associated With Metabolic Risk in Humans

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Background—Although metabolic risk factors are known to cluster in individuals who are prone to developing diabetes mellitus and cardiovascular disease, the underlying biological mechanisms remain poorly understood.

Methods and Results—To identify pathways associated with cardiometabolic risk, we used liquid chromatography/mass spectrometry to determine the plasma concentrations of 45 distinct metabolites and to examine their relation to cardiometabolic risk in the Framingham Heart Study (FHS; n=1015) and the Malmö Diet and Cancer Study (MDC; n=746). We then interrogated significant findings in experimental models of cardiovascular and metabolic disease. We observed that metabolic risk factors (obesity, insulin resistance, high blood pressure, and dyslipidemia) were associated with multiple metabolites, including branched-chain amino acids, other hydrophobic amino acids, tryptophan breakdown products, and nucleotide metabolites. We observed strong associations of insulin resistance traits with glutamine (standardized regression coefficients, −0.04 to −0.22 per 1-SD change in log-glutamine; $P<0.001$), glutamate (0.05 to 0.14; $P<0.001$), and the glutamine-to-glutamate ratio (−0.05 to −0.20; $P<0.001$) in the discovery sample (FHS); similar associations were observed in the replication sample (MDC). High glutamine-to-glutamate ratio was associated with lower risk of incident diabetes mellitus in FHS (odds ratio, 0.79; adjusted $P=0.03$) but not in MDC. In experimental models, administration of glutamine in mice led to both increased glucose tolerance ($P=0.01$) and decreased blood pressure ($P<0.05$).

Conclusions—Biochemical profiling identified circulating metabolites not previously associated with metabolic traits. Experimentally interrogating one of these pathways demonstrated that excess glutamine relative to glutamate, resulting from exogenous administration, is associated with reduced metabolic risk in mice. (***Circulation***. 2012;125:2222-2231.)

Key Words: epidemiology ■ metabolic syndrome ■ metabolomics ■ risk factors

Certain clinical risk factors are known to cluster in individuals who are prone to developing diabetes mellitus and future cardiovascular events.[1] These risk factors are generally associated with insulin resistance and include abdominal obesity, elevated fasting glucose levels, hypertension, and dyslipidemia. The term metabolic syndrome has been used to describe a clustering of these traits among individuals.[2–4] The prevalence of the metabolic syndrome is high, affecting up to 25% of adults in the United States, and is increasing.[5]

Clinical Perspective on p 2231

Pathways underlying metabolic risk factors in humans remain unknown but are likely related to derangements in primary metabolism.[6] Recent advances in liquid chromatog-

raphy tandem mass spectrometry allow the acquisition of high-throughput profiles of the metabolic status of whole organisms (eg, metabolomics), providing a comprehensive assessment of molecules that are substrates or products of metabolic pathways.[7–11] Performing metabolite profiling in individuals with metabolic disorders could elucidate potential roles for specific metabolites in the development of metabolic disease and its sequelae.[12–15] Therefore, we performed metabolite profiling in 2 community-based cohorts comprising individuals spanning a spectrum of metabolic risk. We then performed focused experimental studies in an animal model to test whether interventions aimed at modifying an adverse metabolite profile could attenuate manifestations of metabolic disease.

Methods

Human Study Samples

All human study protocols were approved by the Institutional Review boards of Boston University Medical Center, Massachusetts General Hospital, and Lund University (Sweden). All study participants provided written informed consent. The Framingham Heart Study (FHS) offspring cohort was formed in 1971 with the enrollment of 5124 individuals in a community-based longitudinal cohort study.[16] Of the 2413 attendees who were free of diabetes mellitus and cardiovascular disease at the fifth examination cycle (1991–1995), 650 had metabolite profiling performed as part of 2 nested case-control studies designed to investigate predictors of diabetes mellitus and cardiovascular disease; an additional 365 randomly selected individuals also had metabolite profiling performed.[17] Thus, 1015 individuals were eligible for the present analyses.

Findings in FHS were examined for replication in the Malmö Diet and Cancer Study (MDC), an investigation of 6103 individuals who were originally enrolled between 1991 and 1996 as part of a longitudinal population-based epidemiological cohort. Of the 4577 participants who had complete covariate data and were free of diabetes mellitus and cardiovascular disease at the original examination, 746 had metabolite profiling performed as part of a nested case-control study modeled after that in FHS; these individuals make up the replication sample for the present analyses.

Clinical and Dietary Assessment

All FHS and MDC participants underwent a baseline examination in addition to longitudinal surveillance for incident diabetes mellitus, as described in the Methods section in the online-only Data Supplement.

Metabolite Profiling

For blood specimens collected from the FHS and MDC study participants, profiles of plasma metabolites were obtained as previously described[17] (see the Methods section in the online-only Data Supplement for details).

Murine Studies

Weight-matched C57Bl6 mice were obtained at 7 weeks of age from The Jackson Laboratories (Bar Harbor, ME) and were housed in pairs with free access to water. Details on the experimental models of glucose tolerance and blood pressure response are provided in the Methods section in the online-only Data Supplement.

Statistical Analyses

All metabolite values were natural logarithmically transformed because of their nonnormal distribution and then standardized (to mean=0, SD=1) within each cohort (FHS and MDC). Age- and sex-adjusted Pearson correlation coefficients were estimated to determine correlations between metabolites known to cluster within well-defined groups (amino acids, urea cycle metabolites, etc.) in each study sample. Regression analyses were performed in each study sample to examine the relation of each metabolite (predictor variable) with each clinical metabolic trait (response variables): body mass index (BMI), waist circumference, fasting glucose, log fasting insulin, log homeostasis model assessment of insulin resistance (HOMA), systolic blood pressure (SBP), diastolic blood pressure (DBP), log triglycerides, and high-density lipoprotein (HDL) cholesterol. We analyzed each trait against each metabolite with individual regressions, adjusting for sex and age. In secondary analyses, we adjusted for BMI in addition to age and sex. Regression analyses were performed with mixed linear models[18] that accounted for the sampling algorithm. Given the 45 metabolites analyzed, we used a Bonferroni-corrected P value threshold of 0.001 (0.05/45) to account for the number of metabolites analyzed. Because the majority of metabolites were correlated within well-defined biological groups (amino acids, urea cycle metabolites, etc.), this correction was conservative.

We estimated statistical power to detect associations in the FHS and MDC samples. At the specified significance threshold of 0.001, we had >80% power to detect association of a trait with a metabolite if the true partial correlation was at least 0.129 (FHS) or 0.151 (MDC).

In tertiary analyses, we analyzed the relation of select metabolites (based on cross-sectional analyses results) with risk of future diabetes mellitus in the FHS sample of individuals who were identified as diabetes cases or randomly selected controls (n=601), all of whom were free of diabetes mellitus at baseline. These analyses were performed with logistic regression models adjusted for age, sex, BMI, and baseline fasting glucose.

Analyses of data from the experimental models of glucose tolerance and blood pressure response are described in the Methods section in the online-only Data Supplement.

All analyses were performed with SAS software version 9.1.3 (SAS Institute, Cary, NC).

Results

Characteristics of the human study sample, including participants of the FHS and MDC, are shown in Table 1 and Table I in the online-only Data Supplement, respectively. In FHS, the prevalence of obesity (BMI $\geq$30 kg/m^2) was 31%, and 45% of individuals met the criteria for metabolic syndrome. In the FHS sample, mean Pearson correlations within groups of related analytes were moderate to high for branched-chain amino acids (age- and sex-adjusted r=0.82), the larger group of hydrophobic amino acids (r=0.45), and urea cycle metabolites (r=0.46).

Analytes Associated With Metabolic Traits

In regression analyses adjusted for age and sex (Figure 1 and Table 2), multiple analytes demonstrated significant associations (P<0.001) with $\geq$3 categories of clinical metabolic traits, including body size (BMI or waist circumference), glucose and insulin metabolism (fasting glucose, insulin, or HOMA), SBP or DBP, and lipid abnormalities (HDL cholesterol or triglycerides). Individuals with metabolic traits had highly significant elevations in the branched-chain amino acids (leucine, isoleucine, and valine) and other hydrophobic amino acids, including alanine and the aromatic amino acids (phenylalanine and tyrosine). We identified additional metab-

Table 1.   Characteristics of the Framingham Heart Study Participants (n=1015)

Age, y	56±9
Women, %	47
Body mass index, kg/m^2	28.3±5.0
Waist circumference, cm	95.6±13.8
Systolic blood pressure, mm Hg	129±18
Diastolic blood pressure, mm Hg	76±10
Hypertension, %	41
Serum triglycerides, mg/dL	156±105
Serum HDL, mg/dL	48±14
Fasting glucose, mg/dL	98±10
Fasting insulin, uU/mL	10±8
Metabolic syndrome, %	45
HOMA	2.5±2.2
Insulin resistance, %*	33

HDL indicates high-density lipoprotein; HOMA, homeostasis model assessment of insulin resistance. Values are shown as mean±SD when appropriate.

*Defined as having a HOMA value >75th percentile of the derivation sample.

olites not previously associated with an adverse metabolic profile, including tryptophan breakdown products (kynurenic acid, anthranilic acid) and nucleotide metabolites (xanthosine, n-carbamoyl-β-alanine). Notably, the majority of these analytes were not correlated with fasting glucose.

After adjustment for BMI in addition to age and sex, several metabolites remained significantly associated with multiple metabolic traits, including blood pressure. These metabolites included glutamine, glutamate, proline (a derivative of glutamate), and several hydrophobic amino acids (alanine, tyrosine). The hydrophobic amino acids have been linked to insulin resistance in prior studies, as has the metabolite glutamate.[19] In contrast, the association of glutamine with multiple metabolic risk factors in humans has not been reported previously. After further adjustment for BMI in addition to age and sex, glutamine was inversely associated with insulin, HOMA, SBP, DBP, and log triglycerides and positively associated with HDL ($P<0.0001$ to $P=0.008$; Table 3). Conversely, glutamate was positively associated with insulin, HOMA, SBP, DBP, and log triglycerides and inversely associated with HDL ($P<0.0001$ to $P=0.024$). Because glutamine and glutamate are in a precursor-product relationship, we also examined the glutamine-to-glutamate ratio (Figures I–III in the online-only Data Supplement). In age- and sex-adjusted analyses, the glutamine-to-glutamate ratio was inversely associated with BMI, insulin, HOMA, SBP, DBP, and log triglycerides and positively associated with HDL ($P<0.0001$ to $P=0.024$; Table 3).

In analyses of effect modification by sex on associations with lipid traits, there was no significant sex interaction on the relation of log triglycerides with glutamine, glutamate, or their ratio ($P>0.37$) in the FHS or MDC cohort. There was also no significant sex interaction on the relation of HDL with glutamine ($P>0.15$), although borderline-significant sex interactions were noted for the association of HDL with

glutamate ($P=0.04$) and the ratio ($P=0.048$). In sex-specific analyses, the directionality of relations between these metabolites and HDL was similar but attenuated in men compared with women (Tables II and III in the online-only Data Supplement). In analyses that included additional adjustment for dietary glutamine intake and physical activity, results remained unchanged (data not shown).

We repeated the analyses of glutamate and glutamine in the MDC cohort (Table 4 and the Results section, Table III, and Figure IV in the online-only Data Supplement). In age- and sex-adjusted regression analyses (Table 4), glutamate was significantly associated with BMI, waist circumference, fasting glucose, insulin, HOMA, and log triglycerides and inversely associated with HDL ($P<0.0001$ to $P=0.002$). Glutamine was inversely associated with BMI, waist circumference, fasting glucose, insulin, and HOMA ($P=0.001$ to $P=0.02$). The glutamine-to-glutamate ratio was inversely associated with BMI, waist circumference, fasting glucose, insulin, HOMA, and log triglycerides and positively associated with HDL ($P<0.0001$ to $P=0.0002$). These associations remained significant in analyses that also adjusted for BMI (Table 4 and Table III in the online-only Data Supplement).

Relation of Glutamine and Precursor Amino Acids to Incident Diabetes Mellitus in Humans

In a sample of 601 participants from the FHS cohort who were free of diabetes mellitus at baseline, we examined the relation of glutamine, glutamate, and the glutamine-to-glutamate ratio with incident diabetes mellitus over 12 years. Multivariable logistic regression analyses revealed that glutamine (adjusted odds ratio, 0.83; 95% confidence interval, 0.68–1.02; $P=0.08$), glutamate (odds ratio, 1.29; 95% confidence interval, 1.04–1.60; $P=0.02$), and the glutamine-to-glutamate ratio (odds ratio, 0.79; 95% confidence interval, 0.64–0.98; $P=0.03$) were associated with the risk of diabetes mellitus after adjustment for age, sex, BMI, and baseline fasting glucose. The glutamine-to-glutamate ratio was only weakly correlated with the branched-chain amino acids, which we have previously identified as predictors of incident diabetes mellitus ($r=-0.25$ with isoleucine, $r=-0.15$ with leucine, and $r=-0.07$ with valine).[17] Accordingly, the glutamine-to-glutamate ratio was associated with decreased risk for future diabetes mellitus even after adjustment for the branched-chain amino acids (data not shown). In a sample of 409 participants from the MDC cohort free of diabetes mellitus at baseline, glutamine was inversely associated with incident diabetes mellitus as captured in a registry database (odds ratio, 0.81; 95% confidence interval, 0.67–0.99; $P=0.039$); however, relations of glutamate ($P=0.31$) and the glutamine-to-glutamate ratio ($P=0.91$) were nonsignificant.

Amino Acid Administration and Metabolic Phenotypes in Mice

To test the hypothesis that glutamine or glutamate administration modulates glucose tolerance, we performed a dietary intervention study in mice. Three groups of C57Bl6 mice received 1 of 3 diets for 8 weeks: glutamine plus standard
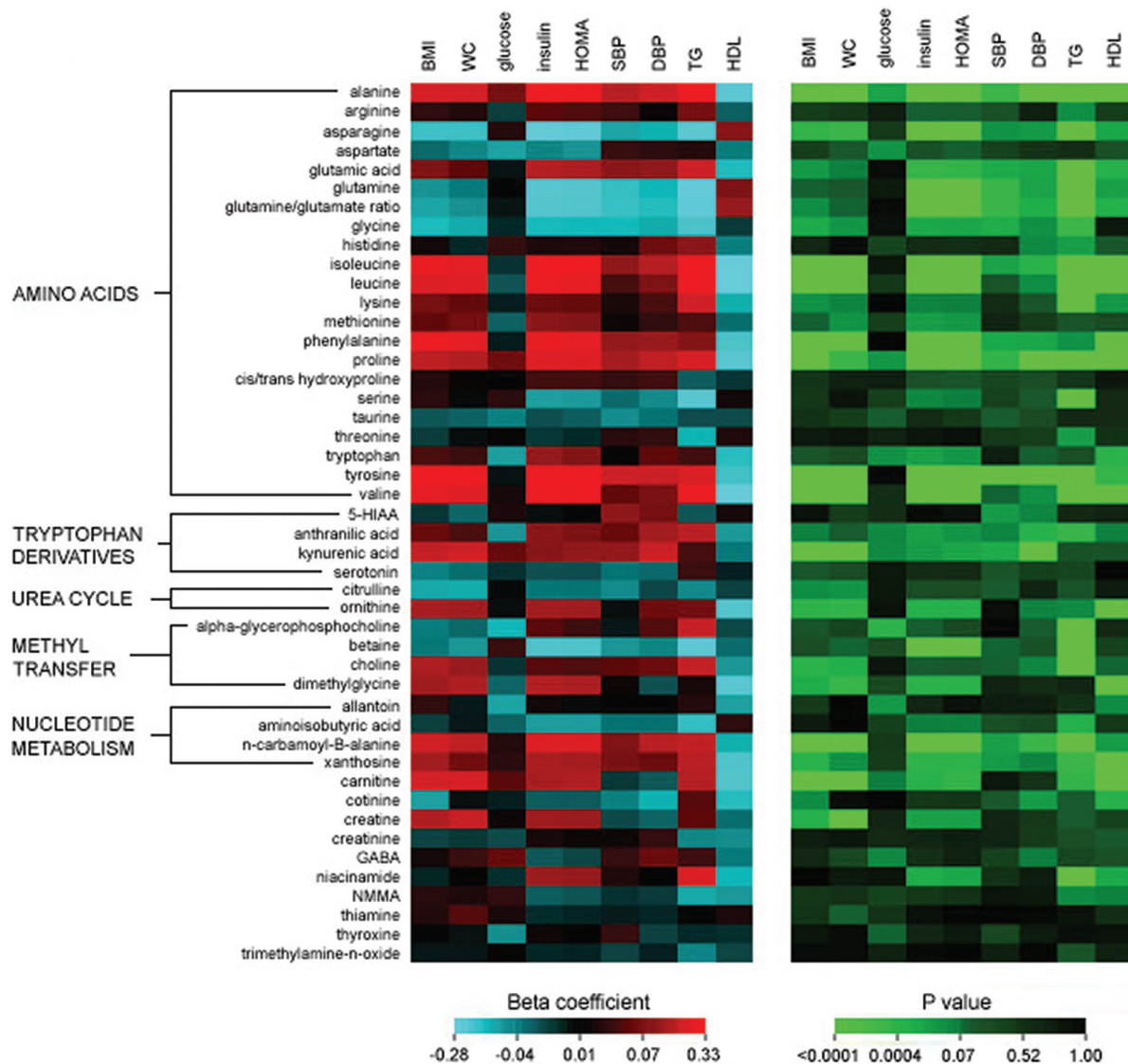
Figure 1. β Coefficients and *P* values generated from age- and sex-adjusted regression analyses of the relation of each metabolite (standardized and log transformed) with insulin resistance phenotypes and metabolic traits (standardized) in the Framingham Heart Study sample. BMI indicates body mass index; WC, waist circumference; HOMA, homeostasis model assessment; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; HDL, high-density lipoprotein; 5-HIAA, 5-Hydroxyindoleacetic acid; GABA, gamma-aminobutyric acid; and NMMA, N-monomethyl-L-arginine.

chow (n=10), glutamate plus standard chow (n=10), and standard chow alone (n=10). Baseline weight and fasting glucose did not differ significantly among the 3 groups. There was no significant difference in the change in weight (*P*=0.19) or total caloric intake (*P*=0.25) across the 3 groups. At the end of the dietary intervention and after a 6-hour fast, all animals received a glucose load injected into the peritoneal cavity (intraperitoneal glucose tolerance test). Compared with mice in the control and glutamate groups, mice in the glutamine group had the lowest plasma glucose levels measured at each time point after glucose administration. Total glucose excursions, represented by the area under the curve of serial glucose measures, are shown in Figure 2A and were significantly lower in the glutamine group compared with the other groups (*P*=0.01).

At the end of the dietary intervention, metabolite profiling confirmed that circulating levels of glutamine and glutamate

were significantly higher in the glutamine- and glutamate-fed mice, respectively, compared with the control group. Metabolite profiling also demonstrated that several analytes had a strong inverse correlation with directly measured levels of glutamine, including the branched-chain amino acids and other hydrophobic amino acids: isoleucine (*r*=−0.65, *P*=0.002), leucine (*r*=−0.73, *P*<0.001), valine (*r*=−0.62, *P*=0.003), tryptophan (*r*=−0.62, *P*=0.003), and tyrosine (*r*=−0.68, *P*<0.001). On the other hand, there was a strong positive correlation between glutamine and citrulline: *r*=0.77 (*P*<0.0001). There were significantly higher plasma concentrations of both glutamine (*P*<0.01) and citrulline (*P*=0.01) in the glutamine-fed mice compared with the control group (Figure 2B).

To test the hypothesis that glutamine administration modulates blood pressure, we performed intraperitoneal injection of either glutamine plus saline or saline alone (vehicle control) in 2 groups of 10 mice. Serial blood pressure

Table 2.   Associations of Metabolites With Insulin Resistance Phenotypes and Metabolic Traits in the Framingham Heart Study

	Regression Coefficient (SE)*								
	BMI	WC	Glucose	Insulin	HOMA	SBP	DBP	Log TG	HDL
5-HIAA	−0.01 (0.04)	−0.03 (0.03)	0.02 (0.03)	0.00 (0.04)	0.01 (0.04)	0.09 (0.03)†	0.07 (0.04)†	−0.02 (0.04)	0.02 (0.03)
Alanine	0.16 (0.03)‖	0.15 (0.03)‖	0.07 (0.03)‡	0.25 (0.03)‖	0.25 (0.03)‖	0.11 (0.03)§	0.14 (0.03)‖	0.33 (0.03)‖	−0.17 (0.03)‖
Allantoin	0.03 (0.03)	0.00 (0.03)	−0.07 (0.03)†	0.02 (0.03)	0.01 (0.03)	0.01 (0.03)	0.01 (0.03)	0.02 (0.03)	−0.06 (0.03)†
a-Glycerophosphocholine	−0.04 (0.03)	−0.03 (0.03)	−0.08 (0.02)§	0.05 (0.03)	0.03 (0.03)	0.00 (0.03)	0.05 (0.03)	0.14 (0.03)‖	−0.02 (0.03)
aminoisobutyric acid	−0.01 (0.03)	0.00 (0.03)	−0.03 (0.03)	−0.06 (0.03)	−0.06 (0.03)	−0.04 (0.03)	−0.03 (0.03)	−0.10 (0.03)‡	0.02 (0.03)
Anthranilic acid	0.06 (0.03)	0.04 (0.03)	−0.05 (0.02)†	0.08 (0.03)‡	0.07 (0.03)†	0.06 (0.03)†	0.10 (0.03)‡	0.11 (0.03)§	−0.06 (0.03)
Arginine	0.03 (0.03)	0.02 (0.03)	−0.01 (0.03)	0.05 (0.03)	0.05 (0.03)	0.04 (0.03)	0.01 (0.03)	0.07 (0.03)†	−0.03 (0.03)
Asparagine	−0.10 (0.03)§	−0.10 (0.03)§	0.02 (0.02)	−0.22 (0.03)‖	−0.20 (0.03)‖	−0.07 (0.03)†	−0.08 (0.03)†	−0.19 (0.03)‖	0.08 (0.03)‡
Aspartate	−0.03 (0.03)	−0.04 (0.03)	−0.07 (0.03)†	−0.04 (0.03)	−0.05 (0.03)	0.03 (0.03)	0.02 (0.03)	0.02 (0.03)	−0.04 (0.03)
Betaine	−0.04 (0.03)	−0.05 (0.03)	0.03 (0.03)	−0.14 (0.03)‖	−0.14 (0.03)‖	−0.05 (0.03)	−0.04 (0.03)	−0.19 (0.03)‖	−0.03 (0.03)
Carnitine	0.15 (0.03)‖	0.16 (0.03)‖	0.05 (0.03)	0.10 (0.03)‡	0.11 (0.03)§	−0.01 (0.03)	−0.02 (0.03)	0.11 (0.03)§	−0.15 (0.03)‖
Choline	0.10 (0.03)§	0.09 (0.03)‡	−0.01 (0.03)	0.05 (0.03)	0.04 (0.03)	0.05 (0.03)	0.06 (0.03)†	0.13 (0.03)‖	−0.05 (0.03)
Cis/trans hydroxyproline	0.02 (0.03)	0.01 (0.03)	0.01 (0.02)	0.04 (0.03)	0.04 (0.03)	0.03 (0.03)	0.04 (0.03)	−0.02 (0.03)	−0.01 (0.03)
Citrulline	−0.07 (0.03)†	−0.07 (0.03)†	0.01 (0.03)	−0.04 (0.03)	−0.04 (0.03)	−0.04 (0.03)	−0.01 (0.03)	−0.04 (0.03)	−0.01 (0.03)
Cotinine	−0.07 (0.03)†	0.00 (0.03)	0.00 (0.02)	−0.02 (0.03)	−0.02 (0.03)	−0.04 (0.03)	−0.08 (0.03)‡	0.05 (0.03)	−0.09 (0.03)‡
Creatine	0.11 (0.03)‡	0.14 (0.03)‖	0.01 (0.03)	0.10 (0.03)‡	0.09 (0.03)‡	−0.02 (0.03)	−0.03 (0.03)	0.05 (0.04)	−0.03 (0.03)
Creatinine	−0.01 (0.03)	−0.01 (0.03)	−0.02 (0.03)	0.02 (0.03)	0.01 (0.03)	0.01 (0.03)	0.03 (0.03)	−0.04 (0.04)	−0.05 (0.03)
Dimethylglycine	0.10 (0.03)‡	0.11 (0.03)§	−0.03 (0.03)	0.11 (0.03)§	0.10 (0.03)‡	0.01 (0.03)	−0.02 (0.03)	0.02 (0.03)	−0.17 (0.03)‖
GABA	0.02 (0.03)	0.03 (0.03)	0.06 (0.03)†	−0.02 (0.03)	−0.01 (0.03)	0.03 (0.03)	0.06 (0.03)	0.04 (0.03)	−0.04 (0.03)
Glycine	−0.09 (0.03)‡	−0.07 (0.03)†	0.00 (0.03)	−0.09 (0.03)‡	−0.09 (0.03)‡	−0.09 (0.03)‡	−0.07 (0.03)†	−0.12 (0.03)§	−0.01 (0.03)
Histidine	0.02 (0.03)	0.00 (0.03)	0.03 (0.03)	0.02 (0.03)	0.02 (0.03)	0.02 (0.03)	0.07 (0.03)†	0.08 (0.03)‡	−0.04 (0.03)
Isoleucine	0.22 (0.03)‖	0.21 (0.03)‖	−0.01 (0.03)	0.26 (0.03)‖	0.25 (0.03)‖	0.09 (0.03)‡	0.11 (0.03)‡	0.29 (0.03)‖	−0.28 (0.03)‖
Kynurenic acid	0.13 (0.03)‖	0.14 (0.03)‖	0.05 (0.03)†	0.08 (0.03)‡	0.09 (0.03)‡	0.09 (0.03)‡	0.14 (0.03)‖	0.04 (0.03)	−0.04 (0.03)
Leucine	0.19 (0.03)‖	0.19 (0.03)‖	−0.02 (0.03)	0.23 (0.03)‖	0.22 (0.03)‖	0.04 (0.03)	0.08 (0.03)†	0.27 (0.03)‖	−0.25 (0.03)‖
Lysine	0.07 (0.03)†	0.06 (0.03)	0.00 (0.03)	0.06 (0.03)	0.06 (0.03)	0.02 (0.03)	0.04 (0.03)	0.14 (0.03)‖	−0.07 (0.03)†
Methionine	0.05 (0.03)	0.07 (0.03)†	−0.03 (0.03)	0.08 (0.03)‡	0.08 (0.03)†	0.01 (0.03)	0.03 (0.03)	0.04 (0.03)	−0.03 (0.03)
n-Carbamoyl-β-alanine	0.15 (0.03)‖	0.12 (0.03)‖	0.02 (0.03)	0.19 (0.03)‖	0.19 (0.03)‖	0.08 (0.03)‡	0.11 (0.03)§	0.13 (0.03)‖	−0.08 (0.03)‡
Niacinamide	0.00 (0.03)	0.01 (0.03)	−0.01 (0.03)	0.09 (0.03)‡	0.09 (0.03)‡	0.02 (0.03)	0.01 (0.03)	0.18 (0.03)‖	−0.08 (0.03)‡
NMMA	0.02 (0.03)	0.02 (0.03)	0.03 (0.03)	−0.02 (0.03)	−0.02 (0.03)	−0.01 (0.03)	−0.01 (0.03)	−0.07 (0.03)†	−0.05 (0.03)
Ornithine	0.10 (0.03)§	0.10 (0.03)§	0.00 (0.03)	0.10 (0.03)‡	0.10 (0.03)‡	0.00 (0.03)	0.06 (0.03)	0.07 (0.03)†	−0.16 (0.03)‖
Phenylalanine	0.23 (0.03)‖	0.21 (0.03)‖	0.00 (0.03)	0.21 (0.03)‖	0.21 (0.03)‖	0.09 (0.03)‡	0.09 (0.03)‡	0.08 (0.03)†	−0.13 (0.03)‖
Proline	0.12 (0.03)‖	0.11 (0.03)§	0.06 (0.03)†	0.19 (0.03)‖	0.19 (0.03)‖	0.10 (0.03)§	0.12 (0.03)‖	0.17 (0.03)‖	−0.17 (0.03)‖
Serine	0.02 (0.03)	0.01 (0.03)	0.02 (0.03)	−0.06 (0.03)†	−0.06 (0.03)	−0.03 (0.03)	−0.04 (0.03)	−0.17 (0.03)‖	0.02 (0.03)
Serotonin	−0.04 (0.03)	−0.03 (0.03)	−0.01 (0.02)	−0.02 (0.03)	−0.02 (0.03)	−0.04 (0.03)	−0.03 (0.03)	0.03 (0.03)	0.00 (0.03)
Taurine	−0.02 (0.03)	−0.03 (0.03)	−0.04 (0.03)	−0.02 (0.03)	−0.02 (0.03)	−0.04 (0.03)	−0.04 (0.03)	−0.02 (0.03)	−0.02 (0.03)
Thiamine	0.02 (0.03)	0.05 (0.03)	0.02 (0.02)	−0.01 (0.03)	0.00 (0.03)	0.00 (0.03)	0.00 (0.03)	0.01 (0.03)	0.02 (0.03)
Threonine	−0.01 (0.03)	0.01 (0.03)	0.01 (0.02)	−0.01 (0.03)	0.00 (0.03)	0.02 (0.03)	0.03 (0.03)	−0.08 (0.03)‡	0.02 (0.03)
Thyroxine	0.01 (0.03)	0.00 (0.03)	−0.05 (0.03)	0.02 (0.03)	0.01 (0.03)	0.03 (0.03)	−0.01 (0.03)	0.00 (0.03)	−0.01 (0.03)
Trimethylamine-N-oxide	0.00 (0.03)	0.00 (0.03)	0.01 (0.03)	0.00 (0.03)	0.00 (0.03)	−0.01 (0.03)	0.00 (0.03)	−0.04 (0.03)	−0.01 (0.03)
Tryptophan	0.04 (0.03)	0.03 (0.03)	−0.06 (0.03)†	0.09 (0.03)‡	0.08 (0.03)†	0.01 (0.03)	0.05 (0.03)	0.04 (0.03)	−0.10 (0.03)‡
Tyrosine	0.26 (0.03)‖	0.24 (0.03)‖	0.00 (0.03)	0.25 (0.03)‖	0.24 (0.03)‖	0.14 (0.03)†	0.14 (0.03)‖	0.18 (0.03)‖	−0.11 (0.03)§
Valine	0.21 (0.03)‖	0.20 (0.03)‖	0.02 (0.03)	0.24 (0.03)‖	0.23 (0.03)‖	0.05 (0.03)	0.07 (0.03)†	0.23 (0.03)‖	−0.24 (0.03)‖
Xanthosine	0.10 (0.03)‡	0.07 (0.03)†	0.02 (0.03)	0.10 (0.03)§	0.10 (0.03)§	0.07 (0.03)†	0.06 (0.03)	0.12 (0.03)§	−0.15 (0.03)‖
Glutamine/isoleucine ratio	−0.10 (0.03)‡	−0.09 (0.03)‡	0.01 (0.02)	−0.22 (0.03)‖	−0.21 (0.03)‖	−0.12 (0.03)†	−0.11 (0.03)§	−0.27 (0.03)‖	0.14 (0.03)‖
Glutamine/leucine ratio	−0.09 (0.03)‡	−0.08 (0.03)‡	0.01 (0.02)	−0.21 (0.03)‖	−0.20 (0.03)‖	−0.11 (0.03)§	−0.10 (0.03)‡	−0.27 (0.03)‖	0.13 (0.03)‖
Glutamine/valine ratio	−0.09 (0.03)‡	−0.08 (0.03)‡	0.00 (0.02)	−0.21 (0.03)‖	−0.21 (0.03)‖	−0.11 (0.03)†	−0.10 (0.03)‡	−0.26 (0.03)‖	0.12 (0.03)‖

BMI indicates body mass index; WC, waist circumference; HOMA, homeostasis model assessment of insulin resistance; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; HDL, high-density lipoprotein cholesterol; 5-HIAA, 5-Hydroxindoleacetic acid; GABA, gamma-aminobutyric acid; and NMMA, N-monomethyl-L-arginine.

*Regression coefficients represent the standardized change in dependent variable per 1-SD change in the log-transformed metabolite. For example, a 1-SD increment in log alanine was associated with a 0.25-SD change in insulin or 2 μU/mL. Separate regressions were fitted for each combination of trait (response) and metabolite (predictor), adjusted for age and sex.

†$P<0.05$.

‡$P<0.01$.

§$P<0.001$.

‖$P<0.0001$.

Table 3. Associations of Select Metabolites With Insulin Resistance Phenotypes and Metabolic Traits in the Framingham Heart Study

	Regression Coefficient (P)*								
	BMI	WC	Glucose	Insulin	HOMA	SBP	DBP	Log TG	HDL
Models adjusting for age and sex									
Glutamate	0.08 (0.011)	0.05 (0.07)	0.00 (0.90)	0.11 (0.0004)	0.10 (0.0006)	0.08 (0.003)	0.09 (0.005)	0.14 (<0.0001)	−0.09 (0.001)
Glutamine	−0.05 (0.09)	−0.04 (0.16)	0.01 (0.78)	−0.17 (<0.0001)	−0.16 (<0.0001)	−0.10 (0.0003)	−0.08 (0.005)	−0.22 (<0.0001)	0.08 (0.008)
Glutamine/glutamate ratio	−0.07 (0.024)	−0.05 (0.08)	0.00 (0.91)	−0.15 (<0.0001)	−0.15 (<0.0001)	−0.10 (0.0003)	−0.09 (0.002)	−0.20 (<0.0001)	0.09 (0.002)
Models adjusting for age, sex, and BMI									
Glutamate	...	−0.01 (0.54)	−0.01 (0.84)	0.07 (0.009)	0.07 (0.011)	0.07 (0.016)	0.07 (0.024)	0.13 (<0.0001)	−0.08 (0.008)
Glutamine	...	0.00 (0.86)	0.01 (0.62)	−0.14 (<0.0001)	−0.13 (<0.0001)	−0.09 (0.0012)	−0.07 (0.017)	−0.20 (<0.0001)	0.06 (0.023)
Glutamine/glutamate ratio	...	0.01 (0.69)	0.01 (0.69)	−0.12 (<0.0001)	−0.11 (<0.0001)	−0.09 (0.0017)	−0.08 (0.011)	−0.19 (<0.0001)	0.08 (0.007)

BMI indicates body mass index; WC, waist circumference; HOMA, homeostasis model assessment of insulin resistance; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; and HDL, high-density lipoprotein cholesterol.

*Regression coefficients represent the standardized change in dependent variable per 1-SD change in the log-transformed metabolite. For example, a 1-SD increment in log glutamine was associated with a −0.17-SD change in insulin or −1.4 μU/mL. Separate regressions were fitted for each combination of trait (response) and metabolite (predictor).

measurements were recorded with the CODA noninvasive tail blood pressure system (Kent Scientific, Torrington, CT). SBP (P=0.016), DBP (P=0.037), and mean arterial pressure (P=0.027) were significantly reduced in glutamine-treated mice versus controls (Figure 2C). As in the feeding experiment, levels of both glutamine (P<0.001) and citrulline (P=0.04), a known precursor to nitric oxide, were significantly higher in the glutamine-treated animals (Figure 2D). In a parallel experiment of glutamate administration, no significant difference in blood pressure was observed between the mice given glutamate plus saline versus those given saline alone (data not shown).

Discussion

High-throughput metabolite profiling provides the opportunity to perform a systematic, unbiased investigation of the possible pathways underlying complex phenotypes such as the metabolic syndrome. Therefore, we applied metabolite profiling in 2 community-based cohorts and observed that individuals with metabolic risk factors have higher circulating concentrations of glutamate, branched-chain amino acids, and other amino acid derivatives and lower concentrations of glutamine. We observed this metabolomic signature in association with multiple components of the metabolic syndrome, including central adiposity, glucose intolerance, dyslipidemia, and hypertension. Furthermore, we found that an excess of glutamine relative to glutamate in the circulation was associated with a reduced risk of future diabetes mellitus. Concordant with the findings in humans, we observed that glutamine supplementation improved glucose tolerance and lowered blood pressure in mice. Together, these data suggest that diminished glutamine, particularly in relation to its precursor amino acids, not only is a marker of metabolic risk but also may contribute to the development of metabolic disease. Our study also identified unanticipated associations

Table 4. Associations of Select Metabolites With Insulin Resistance Phenotypes and Metabolic Traits in the Malmö Diet and Cancer Study

	Regression Coefficient (P)*								
	BMI	WC	Glucose	Insulin	HOMA	SBP	DBP	Log TG	HDL
Models adjusting for age and sex									
Glutamate	0.16 (<0.0001)	0.17 (<0.0001)	0.09 (0.002)	0.14 (0.0001)	0.17 (<0.0001)	0.08 (0.031)	0.05 (0.15)	0.23 (<0.0001)	−0.15 (<0.0001)
Glutamine	−0.12 (0.001)	−0.08 (0.006)	−0.07 (0.022)	−0.10 (0.008)	−0.11 (0.006)	−0.05 (0.15)	−0.03 (0.44)	−0.02 (0.52)	0.06 (0.081)
Glutamine/glutamate ratio	−0.19 (<0.0001)	−0.19 (<0.0001)	−0.11 (0.0002)	−0.17 (<0.0001)	−0.20 (<0.0001)	−0.09 (0.010)	−0.06 (0.10)	−0.22 (<0.0001)	0.17 (<0.0001)
Models adjusting for age, sex, and BMI									
Glutamate	...	0.06 (<0.0001)	0.09 (0.004)	0.10 (0.006)	0.12 (0.0006)	0.05 (0.19)	0.02 (0.61)	0.19 (<0.0001)	−0.11 (0.0006)
Glutamine	...	0.00 (0.74)	−0.07 (0.038)	−0.07 (0.069)	−0.07 (0.063)	−0.03 (0.45)	0.00 (0.97)	0.01 (0.76)	0.03 (0.41)
Glutamine/glutamate ratio	...	−0.05 (0.0002)	−0.11 (0.0005)	−0.12 (0.0009)	−0.14 (<0.0001)	−0.05 (0.13)	−0.02 (0.64)	−0.18 (<0.0001)	0.12 (0.0004)

BMI indicates body mass index; WC, waist circumference; HOMA, homeostasis model assessment of insulin resistance; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; and HDL, high-density lipoprotein cholesterol.

*Regression coefficients represent the standardized change in dependent variable per 1-SD change in the log-transformed metabolite. For example, a 1-SD increment in log glutamine was associated with a −0.10-SD change in insulin or −1.0 μU/mL. Separate regressions were fitted for each combination of trait (response) and metabolite (predictor).
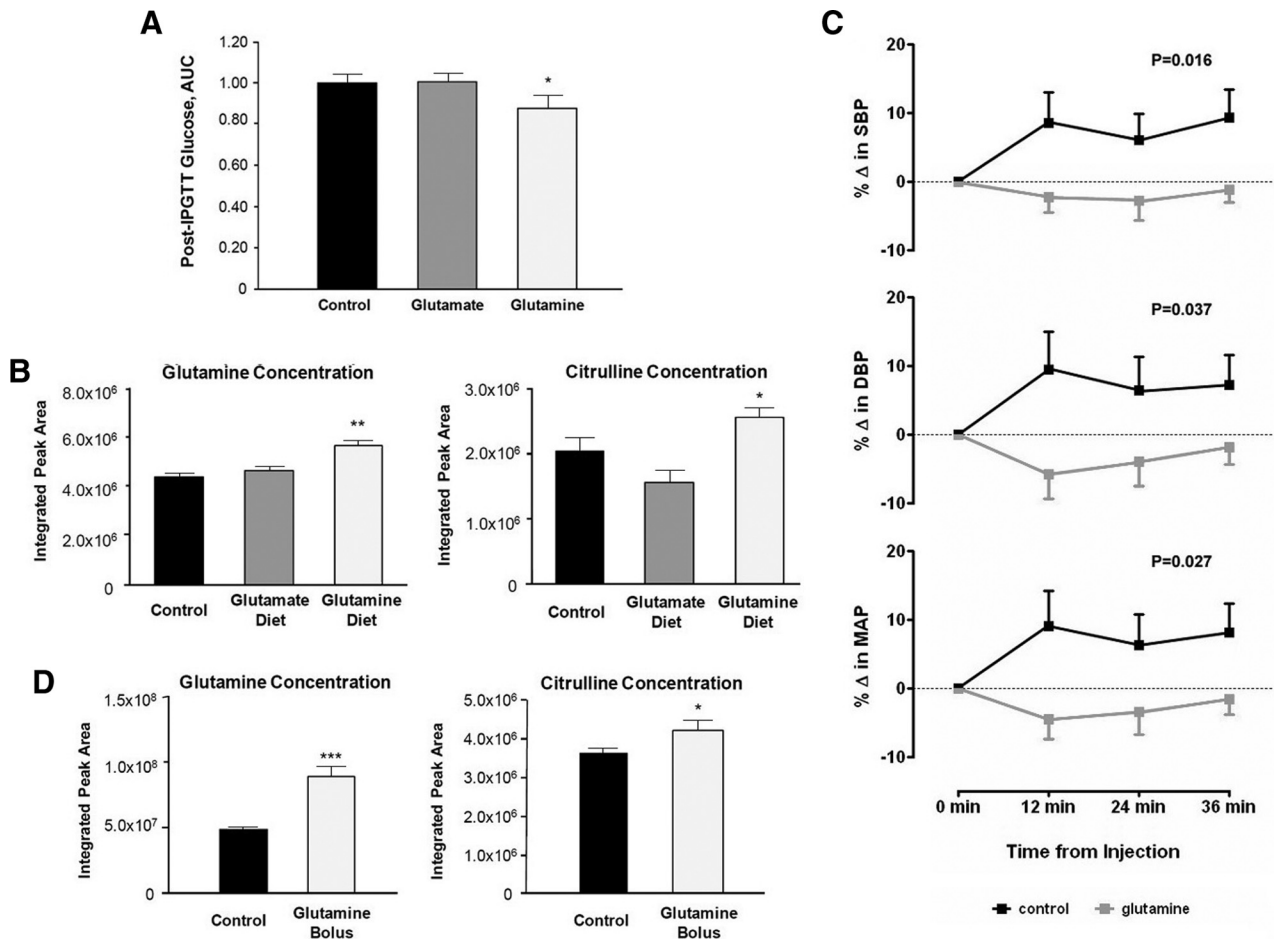
Figure 2. Experimental results. **A**, Mean±SE area under the curve (AUC) of serial glucose levels measured after intraperitoneal glucose tolerance test (IPGTT) in mice treated for 8 weeks with dietary glutamine, glutamate, or standard chow alone (control). **B**, Mean±SE measures of glutamine and citrulline are shown in mice treated with dietary glutamine, glutamate, or standard chow alone (control). **C**, Mean±SE measures of systolic (SBP), diastolic (DBP), and mean (MAP) blood pressure measurements are shown for mice after intraperitoneal injection of either glutamine plus saline (glutamine) or saline alone (control). **D**, Mean±SE measures of glutamine and citrulline are shown for mice after intraperitoneal injection of either glutamine plus saline (glutamine) or saline alone (control).

of tryptophan breakdown products (kynurenic acid, anthranilic acid) and nucleotide metabolites (xanthosine, n-carbamoyl-β-alanine) with cardiometabolic disease.

Several patterns noted in our metabolomic data are consistent with results of prior experimental and physiological studies. Felig and colleagues[19] originally reported that selected amino acids were associated with fasting insulin levels in 10 obese and 10 nonobese individuals. More recent investigations in small samples have highlighted associations of the branched-chain amino acids (isoleucine, leucine, and valine) and other hydrophobic amino acids (eg, alanine, phenylalanine, and tyrosine) with obesity, impaired glucose tolerance, and insulin resistance.[12–15,19] A population study also observed an association of 24-hour urinary excretion of alanine with higher blood pressure.[11] The present investigation extends the prior work by demonstrating robust and reproducible associations of circulating levels of branched-chain amino acids not only with obesity and impaired glucose tolerance but also with dyslipidemia and blood pressure in 2 large human cohorts.

Our metabolomic data also highlight several patterns that have not previously been well described in association with

metabolic disease. Glutamine is best known for its role in providing intermediates to the tricarboxylic acid cycle (anapleurosis) and for its ammonia-carrying capacity, which is critical for maintaining overall nitrogen balance.[20–22] Total body glutamine is depleted in catabolic states such as trauma, critical illness, infection, and sepsis, and parenteral glutamine supplementation in the intensive care setting is associated with reduced morbidity and mortality.[23] These findings have been attributed to the effects of glutamine on the regulation of white cell function, cell volume, response to cytotoxins, and cellular redox potential via glutathione-mediated pathways.[21] We now demonstrate in 2 large human cohorts that glutamine is inversely associated with a wide array of metabolic traits, including hypertension and hypertriglyceridemia, in addition to measures of obesity and insulin resistance. In our data, standard deviation increments in metabolite concentrations were generally associated with meaningful differences in metabolic trait measures (eg, a 1.2- to 2.0-μU/mL difference in plasma insulin). The consistent associations observed between multiple glutamine-related metabolites and metabolic traits further support the hypothesis that glutamine-cycling pathways are prominently involved in the development of metabolic risk.

Interestingly, a prior study of 24 adults with and without diabetes mellitus showed that glutamine supplementation was associated with improved glucose tolerance.[24] In support of the hypothesis that glutamine may be more than a marker of metabolic risk, animal studies have shown that glutamine administration improves glucose and insulin metabolism in the setting of exercise[25] or a high-fat diet.[26] Our experimental data extend these findings by distinguishing the protective effect of glutamine versus glutamate supplementation, showing the corresponding changes in metabolite profiles, and demonstrating that the improvements in glucose tolerance are independent of changes in weight. Furthermore, we found that glutamine administration decreases blood pressure after short-term administration.

Taken together, these data underscore the potentially beneficial effects of glutamine on cardiometabolic risk, which may be due to a number of mechanisms such as enhanced release of glucagon-like peptide 1, externalization of glucose transporter type 4, transcription of insulin-dependent enzymes, pancreatic β-cell insulin secretion, and increased insulin sensitivity of adipose tissue.[24,27,28] The association of glutamine with blood pressure may be further related to direct or indirect effects on the regulation of nitric oxide production,[29–31] as suggested by our finding of strong correlations between glutamine and citrulline, a known precursor to nitric oxide.

The distinct roles of glutamine versus glutamate have not previously been highlighted. Endogenous glutamine is continually synthesized in the skeletal muscle, predominantly from glutamate but also from branched-chain amino acids. Cycling between glutamine and glutamate is regulated by the activity of glutamine synthetase and glutaminase, enzymes that have wide tissue distribution.[21,32] Prior studies have observed an association of dietary glutamate with lower blood pressure[33] but without accounting for its effect on endogenous glutamine levels.[34] A recent investigation of metabolomic profiles in humans observed that obesity and insulin resistance are associated with an analyte identified as both glutamine and glutamate.[12] Our platform distinguishes the 2 analytes, highlighting the unanticipated finding that glutamate may confer adverse metabolic risk whereas glutamine may be protective. In contrast to the likely favorable effects of glutamine, glutamate has been shown to stimulate glucagon release from pancreatic α cells[35] and to increase transamination of pyruvate to alanine, a strong promoter of gluconeogenesis that is abundant in obesity.[12] Glutamate is also well known as a direct precursor to α-ketoglutarate, an intermediate in the Krebs cycle that serves as an energy source for multiple cell types and that exerts anabolic as well as anticatabolic activity.[36]

Several limitations of the present study merit consideration. We used a liquid chromatography tandem mass spectrometry platform that does not provide full coverage of the plasma metabolome. However, our focus on abundant metabolites permitted the measure of 45 analytes; by providing key clinical correlates for a large panel of plasma metabolites, these data complement ongoing efforts to annotate the human metabolome.[37] The present analysis was focused on interrogating the relationship of glutamine (and its precursor analytes) with metabolic traits and potential functional roles. The biology underlying the correlates of other analytes remains the subject of future investigation. A small number of metabolites had coefficients of variation exceeding 20% but were retained in analyses for completeness; the results of analyzing these specific metabolites may have been biased to the null. As a surrogate measure of insulin resistance, HOMA-IR is less precise than measures obtained from the hyperinsulinemic euglycemic glucose clamp technique or the insulin suppression test. Nonetheless, it is regarded as a reasonably reliable surrogate in individuals without severely impaired pancreatic β-cell function and is considered practical for epidemiological studies.[38,39] Because the MDC cohort was relatively small and consisted exclusively of individuals previously identified as having a high baseline risk for developing incident diabetes mellitus or cardiovascular disease, analyses of incident diabetes mellitus in this cohort were limited by sampling in addition to possible ascertainment bias. Thus, additional investigations of the relation of glutamine and glutamate with incident diabetes mellitus are needed in unselected populations. Because many of the metabolites included in the present study were amino acids or amino acid precursors or derivatives, the possible associations of non–amino acid molecules with cardiometabolic risk remain a subject for further investigation. Our clinical cohorts were comprised of predominantly middle-aged to elderly individuals of European ancestry; thus, the generalizability of our findings to younger individuals and other racial/ethnic groups is not yet known.

The present study demonstrates the potential value of integrating metabolomic and clinical data in human cohorts with detailed phenotyping. While highlighting select amino acids of interest, we recognize that our data relating all of the measured metabolites to metabolic traits in a large sample of ambulatory individuals may be of broader scientific interest. Indeed, we submit that several features favor the potential of our data to serve as a resource for future investigations. First, these data demonstrate the feasibility and reproducibility of analyzing the relations of metabolomic with clinical data across 2 separate community-based cohorts. Second, our results provide an important clinical dimension to numerous plasma metabolites, complementing ongoing efforts to annotate the human metabolome.[37] Third, the range of observed associations between metabolites and metabolic traits highlights opportunities for further research aimed at elucidating pathways underlying cardiometabolic disease and identifying potential therapeutic targets. Because metabolites represent intermediate traits that may play functional roles (either adaptive or maladaptive) in disease pathogenesis, future applications of metabolomics technology can provide additional insights into the mechanisms by which established risk factors are associated with clinically important cardiovascular and metabolic outcomes.

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Disclosures

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CLINICAL PERSPECTIVE

Although metabolic risk factors are known to cluster in individuals who are prone to developing diabetes mellitus and cardiovascular disease, the underlying biological mechanisms remain poorly understood. To acquire a more detailed understanding of the biochemical pathways, we applied high-throughput metabolite profiling to samples from 1761 individuals from 2 large, well-characterized clinical cohorts. We observed that the presence of metabolic risk factors (including obesity, insulin resistance, high blood pressure, and dyslipidemia) was significantly associated with variation in select metabolites, including branched-chain amino acids, other hydrophobic amino acids, tryptophan breakdown products, and nucleotide metabolites. We observed particularly strong associations of insulin resistance traits with decreased glutamine and increased glutamate. We followed up these findings in experimental models and demonstrated that glutamine administration in mice resulted in both increased glucose tolerance and decreased blood pressure. Taken together, our clinical and experimental data highlight the glutamine-glutamate metabolic pathway as a potential target for interventions aimed at attenuating metabolic risk in humans. Furthermore, by demonstrating the feasibility and utility of biochemical profiling in large clinical samples, we anticipate that our data could serve as a resource for future studies of the human metabolome and its relevance to cardiovascular and metabolic diseases. Because metabolites represent intermediate traits that may play functional roles (either adaptive or maladaptive) in disease pathogenesis, future applications of metabolomics technology can provide additional insights into the mechanisms by which established risk factors are associated with clinically important outcomes.

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Clinical and Dietary Assessment

In FHS and MDC, all study participants underwent a standardized physical examination and medical history, and routine laboratory tests. The homeostasis model assessment (HOMA) of insulin resistance was calculated according to the standard formula[1] and used as a measure of relative insulin resistance. In FHS, dietary intake of glutamine was estimated using a detailed food frequency questionnaire.[2] Physical activity was assessed using a physical activity index that has been described previously.[3] In the FHS, presence of diabetes was ascertained at each follow-up examination and was defined as a fasting glucose ≥126 mg/dL, or the use of glucose-lowering medication, or a 2-hour glucose ≥200 mg/dL following an oral glucose tolerance test. In the MDC, presence of diabetes at the baseline examination (1991-1996) was defined as a fasting glucose ≥126 mg/dL, or the use of glucose-lowering medication, or self report of a physician diagnosis of diabetes. In MDC, a diagnosis of new-onset diabetes after the baseline examination (until December 2005, mean follow-up time 12.6 years) was based on individual data from 3 registries: the Malmö HbA1c registry (MHR), the nationwide Swedish National Diabetes Registry (NDR),[4] and the regional Diabetes 2000 registry of the Scania region.[5] In the NDR and Diabetes 2000 registries, presence of diabetes required a physician diagnosis based on a fasting glucose ≥ 126 mg/dL measured on two separate occasions.

The study protocols were approved by the Institutional Review Boards of Boston University

Medical Center, Massachusetts General Hospital, and Lund University, Sweden. All participants

provided written informed consent.

Metabolite Profiling

Metabolite profiling was performed using a 4000 QTRAP triple quadrupole mass spectrometer (AB

SCIEX, Foster City, CA), coupled to a multiplexed LC system including two 1200 Series pumps (Agilent

Technologies, Santa Clara, CA) and an HTS PAL autosampler (Leap Technologies, Carrboro, NC) with 2

injection ports and a column selection valve. The two pumps were similarly configured for hydrophobic

interaction chromatography (HILIC) using 150 x 2.1 mm Atlantis HILIC columns (Waters; Milford,

MA). MultiQuant software v1.1 (AB SCIEX, Foster City, CA) was used for automated peak integration

and metabolite peaks also were manually reviewed for quality of integration. Formic acid, ammonium

acetate, LC/MS grade solvents, and valine-d8 were obtained from Sigma-Aldrich (St. Louis, MO), with

the remainder of isotopically-labeled analytical standards obtained from Cambridge Isotope Laboratories,

Inc. (Andover, MA). Internal standard peak areas were monitored for quality control and individual

samples with peak areas differing from the group mean by more than 2 standard deviations were re-

analyzed. Metabolites analyzed were selected based on the following criteria: 1) known structural

identity; 2) distribution across multiple biochemical pathways; 3) reliable measurement using LC/MS in a

high throughput fashion; and, 4) low rate of missingness on our platform (<1%). Of the 45 metabolites

included in the present study, 67% had CV ≤10% and 89% had CV≤20%. For quantification of serotonin

and 5-HIAA, measurement values were missing for 0.4% and 0.9% of the total sample; these values were

coded as missing in analyses of these select metabolites.

Murine Studies

Weight-matched C57Bl6 mice were obtained at 7 weeks of age from Jackson Laboratories (Bar Harbor, ME) and were housed in pairs with free access to water. All animal experiments were performed according to procedures approved by the Institutional Review Board at Massachusetts General Hospital.

In an experimental model of glucose tolerance, 30 mice were randomized to 1 of the following 3 groups: a standard high-fat diet (control); a high-fat diet enriched with glutamic acid (150% concentration of glutamic acid compared to control); and, a high-fat diet enriched with glutamine (150% concentration of glutamine compared to control). The total caloric content and total percentage of protein (23%), carbohydrate (34%), and fat (43%) in the administered diets were not significantly different across animal groups (Research Diets, Brunswick, NJ). Pre-weighed food was administered to each cage, and food intake was determined by grams consumed per day. Food intake and body weights were monitored weekly, and adjustments were made to ensure equivalent calorie intake per animal. After 8 weeks of dietary treatment, and following a 6-hour fast, each group of mice was administered an intra-peritoneal glucose tolerance test (IPGTT), during which a glucose load (1.5 mg/g of body weight; 75 mg/mL of glucose solution) was injected into the peritoneal cavity. Venous blood samples were obtained from the tail vein immediately prior to glucose injection (baseline) and then serially at 30, 60, and 120 minutes following the glucose injection. From these blood samples, collected at serial time points, concentrations of glucose and select metabolites were assayed. Blood glucose levels were determined using a Bayer Contour Glucose Meter (Bayer Healthcare, Mishawaka, IL). Glucose response (and metabolite response) was calculated as area under the curve (AUC) of the serial measurements for each of the 3 groups of mice. An additional blood sample was obtained via cardiac puncture, 48 hours following the glucose testing, for additional metabolite profiling analyses.

In an experimental model of blood pressure response following metabolite challenge, 2 groups of 10 mice each were trained for 2 days prior to a protocol that involved serial blood pressure measurements

following an acute bolus of either glutamine plus saline or saline alone (control). During training, 20 blood pressure measurements were recorded for each animal using the CODA non-invasive tail blood pressure system (Kent Scientific, Torrington, CT). On the day of the experiment, the baseline blood pressure was measured in each mouse. Each animal subsequently received either a peritoneal bolus injection of glutamine dissolved in saline (pH 7.4) at a dose of 1200 mg/kg or saline alone (control). Serial blood pressure measurements were then obtained every 12 minutes for 36 minutes. Five measurements were obtained bracketing each time point. Additionally, a venous blood sample was collected for metabolite profiling analyses from each animal following the final time point.

Statistical Analyses

In the experimental model of glucose tolerance, blood glucose levels were measured at baseline, prior to the glucose injection, and then at serial time points (30, 60, and 120 minutes) following the glucose injection. Glucose response was calculated as area under the curve (AUC) of the serial glucose measurements for each of the 3 groups of mice. We used analysis of variance (ANOVA) to compare AUCs among groups. Metabolite profiles were determined on blood samples collected from each animal, and concentrations of the analytes measured were compared between the glutamine and control group using t-tests. We also determined the Pearson correlation between plasma glutamine and each of the other analytes measured.

In the experimental model of blood pressure response, blood pressure measurements were recorded at serial time points (12, 24, and 36 minutes) following injection of either glutamine plus saline or saline alone (control) in 2 groups of mice. We used repeated measures ANOVA tests to compare change in SBP, DBP, and mean arterial pressure (MAP) from baseline between the 2 groups over the course of the experiment. From metabolite profiling data collected after the acute bolus injection, we used t-tests to compare concentrations of the measured analytes between the 2 groups.

SUPPLEMENTAL RESULTS

Analytes Associated with Metabolic Traits: Sex-Specific Analyses

Given prior reports of possible sex-based differences in the relation of lipids with cardiovascular risk, we tested for the presence of effect modification by sex on the associations of lipid traits with glutamine, glutamate, and the glutamine-glutamate ratio. In both FHS and MDC, there was no significant sex interaction on the relation of log triglycerides with glutamine, glutamate, or the ratio (P>0.37). There was also no significant sex interaction on the relation of HDL with glutamine (P>0.15), although sex interactions were significant for the associations of HDL with glutamate (P<0.037) and the ratio (P<0.048). In sex-specific analyses, the directionality of relations between these metabolites and HDL was similar but attenuated in men compared to women (**Supplemental Tables 2 and 3**).

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Supplemental Table 1. Characteristics of the MDC Study Participants

	MDC (N=746)
Age, years	59±6
Women, %	51
Body mass index, kg/m^2	26.8±4.3
Waist circumference, cm	88.0±13.5
Systolic blood pressure, mmHg	147±19
Diastolic blood pressure, mmHg	90±9
Hypertension, %	76
Serum triglycerides, mg/dL	114 (85, 159)
Serum HDL, mg/dL	50±13
Fasting glucose, mg/dL	92.3±8.5
Fasting insulin, uU/mL	9.0±9.7
Metabolic syndrome, %	33
HOMA	2.1±2.1
Insulin resistance, %*	21

MDC, Malmö Diet and Cancer Study; HOMA, homeostasis model assessment of insulin resistance.
*Defined as having HOMA value >75th percentile of the derivation sample.

Supplemental Table 2. Sex-Specific Associations of Select Metabolites with Lipid Traits in FHS

	Regression Coefficient (*P* value)			
	Log TG		HDL	
	Men	**Women**	**Men**	**Women**
Models adjusting for age				
glutamate	0.16 (0.0005)	0.12 (0.006)	-0.06 (0.055)	-0.13 (0.010)
glutamine	-0.24 (<0.0001)	-0.19 (<0.0001)	0.03 (0.32)	0.14 (0.008)
glutamine/glutamate ratio	-0.23 (<0.0001)	-0.17 (0.0001)	0.05 (0.13)	0.15 (0.004)
Models adjusting for age and BMI				
glutamate	0.14 (0.002)	0.10 (0.012)	-0.05 (0.13)	-0.11 (0.020)
glutamine	-0.22 (<0.0001)	-0.18 (<0.0001)	0.02 (0.55)	0.13 (0.011)
glutamine/glutamate ratio	-0.21 (<0.0001)	-0.16 (0.0003)	0.03 (0.28)	0.13 (0.008)

TG, triglycerides; HDL, high-density lipoprotein cholesterol.
*Regression coefficients represent the standardized change in dependent variable per 1-SD change in the log-transformed metabolite. Separate regressions were fitted for each combination of trait (response) and metabolite (predictor).

Supplemental Table 3. Sex-Specific Associations of Select Metabolites with Lipid Traits in MDC

| | Regression Coefficient (*P* value) | | | |
| | Log TG | | HDL | |
	Men	**Women**	**Men**	**Women**
Models adjusting for age				
glutamate	0.23 (<0.0001)	0.23 (<0.0001)	-0.05 (0.23)	-0.23 (<0.0001)
glutamine	0.00 (0.95)	-0.07 (0.21)	0.05 (0.27)	0.07 (0.17)
glutamine/glutamate ratio	-0.21 (<0.0001)	-0.24 (<0.0001)	0.07 (0.13)	0.24 (<0.0001)
Models adjusting for age and BMI				
glutamate	0.22 (<0.0001)	0.16 (0.0006)	-0.04 (0.39)	-0.17 (0.0004)
glutamine	0.02 (0.76)	-0.01 (0.89)	0.04 (0.43)	0.02 (0.72)
glutamine/glutamate ratio	-0.20 (0.0002)	-0.16 (0.0008)	0.05 (0.28)	0.18 (0.0004)

TG, triglycerides; HDL, high-density lipoprotein cholesterol.
*Regression coefficients represent the standardized change in dependent variable per 1-SD change in the log-transformed metabolite. Separate regressions were fitted for each combination of trait (response) and metabolite (predictor).
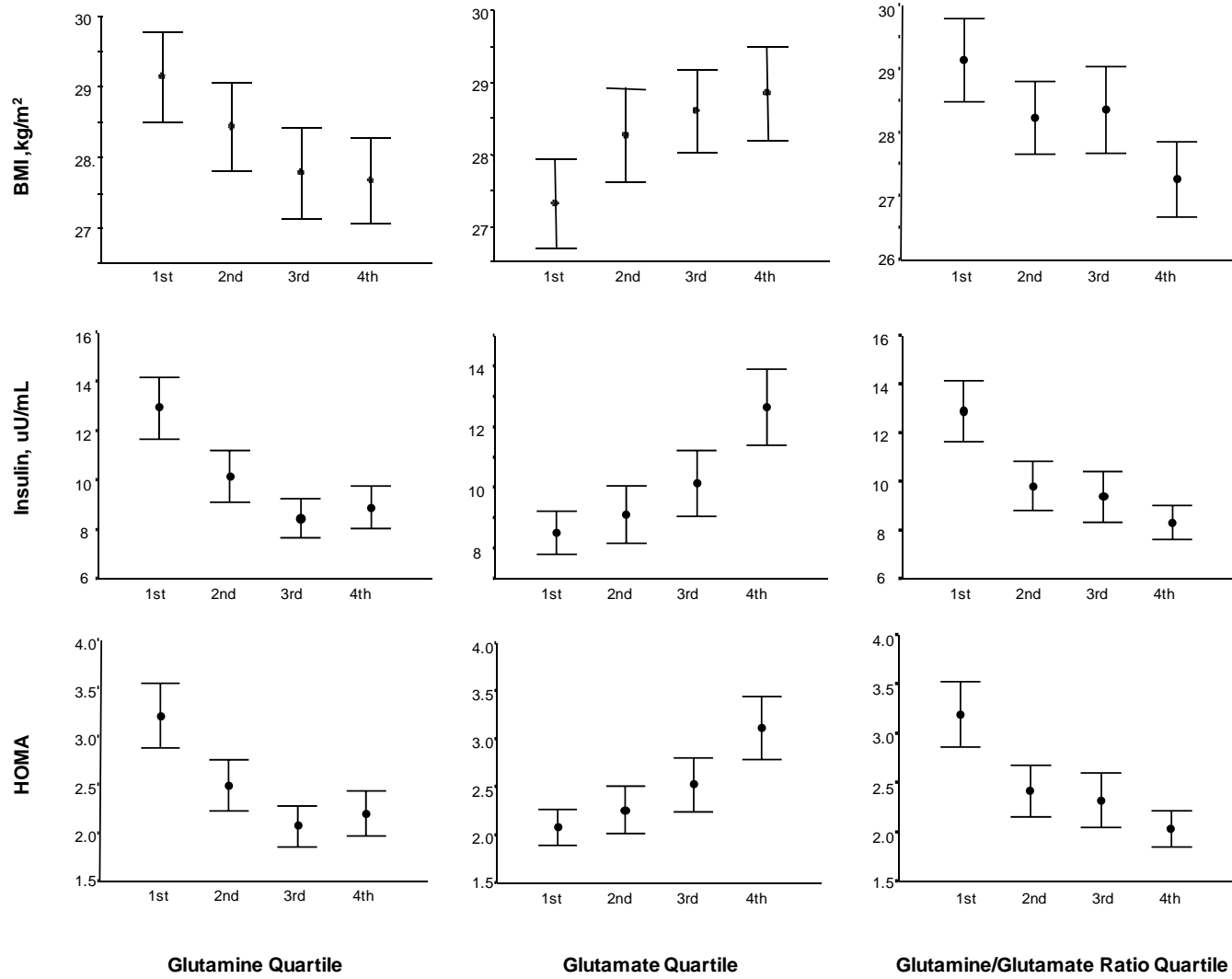
Supplemental Figures Legend

Supplemental Figure 1. Association of Insulin Resistance Phenotypes with Select Metabolites in FHS. Mean (± standard error) values of insulin resistance phenotypes by quartile of glutamine, glutamate, and glutamine/glutamate ratio in the FHS sample.

Supplemental Figure 2. Association of Insulin Resistance Phenotypes with Select Metabolites in MDC. Mean (± standard error) values of insulin resistance phenotypes by quartile of glutamine, glutamate, and glutamine/glutamate ratio in the MDC sample.

Supplemental Figure 3. Association of Blood Pressure Traits with Select Metabolites in FHS. Mean (± standard error) values of blood pressure traits by quartile of glutamine, glutamate, and glutamine/glutamate ratio in the FHS sample.

Supplemental Figure 4. Association of Lipid Traits with Select Metabolites in FHS. Mean (± standard error) values of lipid traits by quartile of glutamine, glutamate, and glutamine/glutamate ratio in the FHS sample.
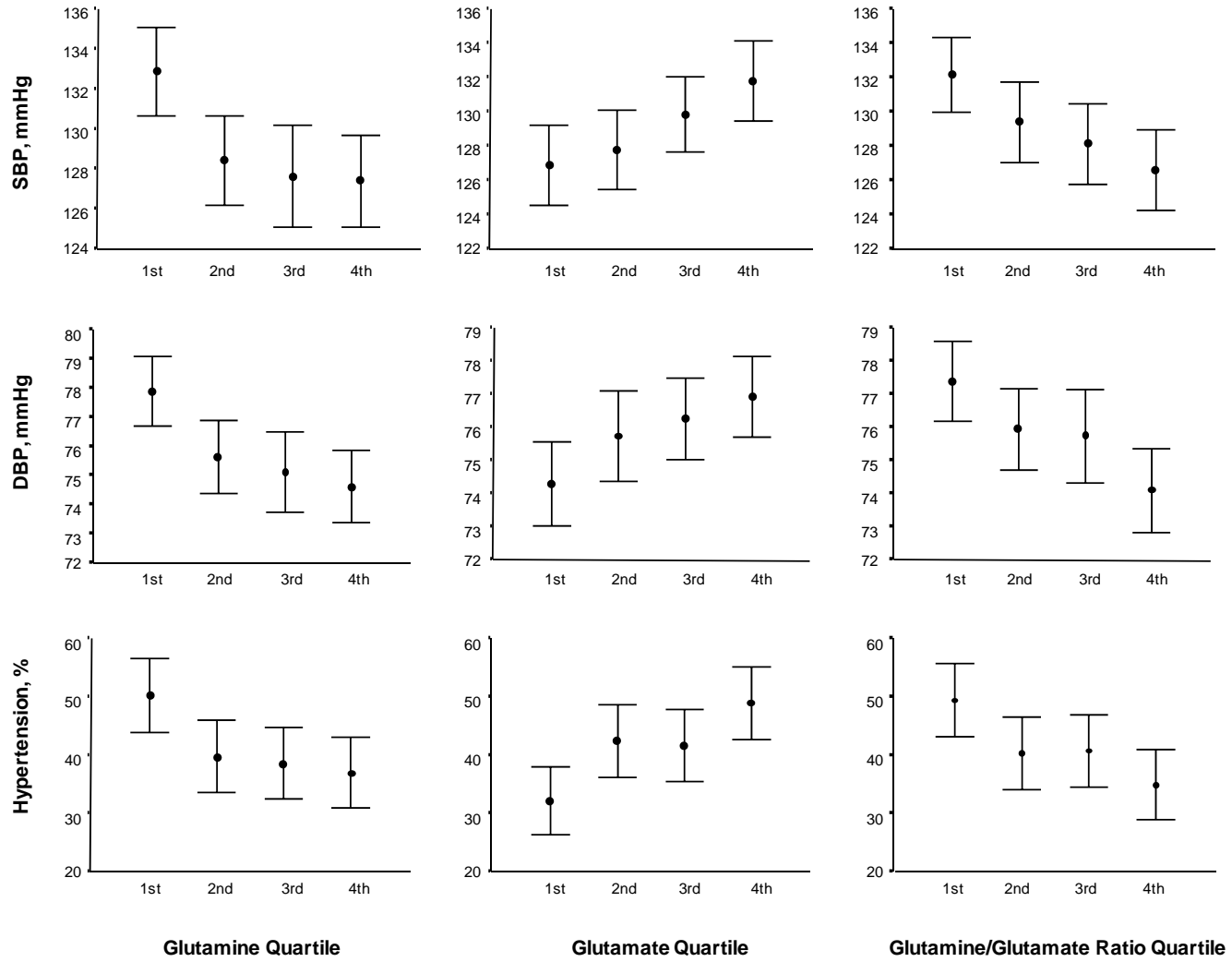
Supplemental Figure 1.

Glutamine Quartile   Glutamate Quartile   Glutamine/Glutamate Ratio Quartile

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Supplemental Figure 2.
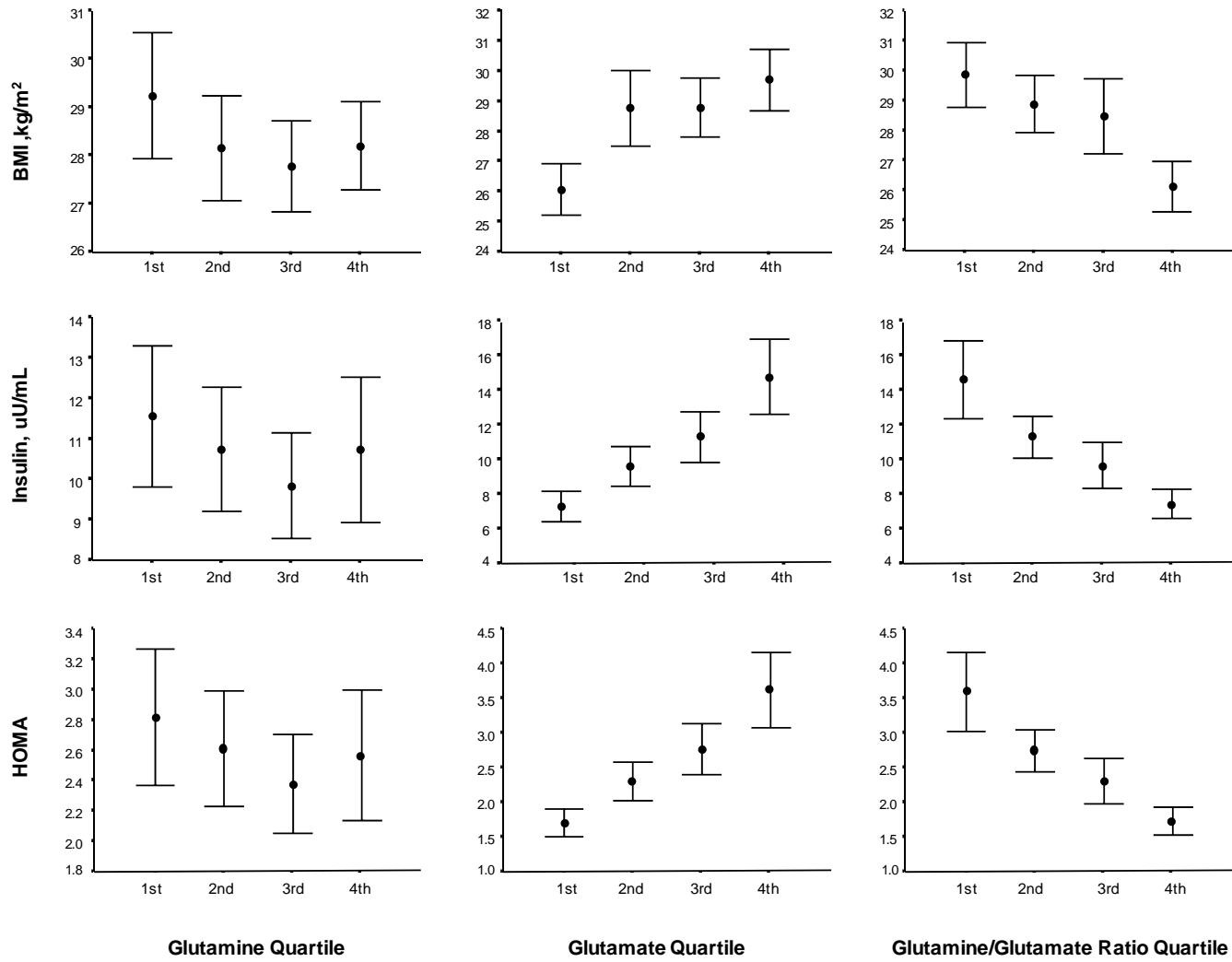
Supplemental Figure 3.

Supplemental Figure 4.