Hyperglycemia and a Common Variant of *GCKR* Are Associated With the Levels of Eight Amino Acids in 9,369 Finnish Men

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We investigated the association of glycemia and 43 genetic risk variants for hyperglycemia/type 2 diabetes with amino acid levels in the population-based Metabolic Syndrome in Men (METSIM) Study, including 9,369 nondiabetic or newly diagnosed type 2 diabetic Finnish men. Plasma levels of eight amino acids were measured with proton nuclear magnetic resonance spectroscopy. Increasing fasting and 2-h plasma glucose levels were associated with increasing levels of several amino acids and decreasing levels of histidine and glutamine. Alanine, leucine, isoleucine, tyrosine, and glutamine predicted incident type 2 diabetes in a 4.7-year follow-up of the METSIM Study, and their effects were largely mediated by insulin resistance (except for glutamine). We also found significant correlations between insulin sensitivity (Matsuda insulin sensitivity index) and mRNA expression of genes regulating amino acid degradation in 200 subcutaneous adipose tissue samples. Only 1 of 43 risk single nucleotide polymorphisms for type 2 diabetes or hyperglycemia, the glucose-increasing major C allele of rs780094 of *GCKR*, was significantly associated with decreased levels of alanine and isoleucine and elevated levels of glutamine. In conclusion, the levels of branched-chain, aromatic amino acids and alanine increased and the levels of glutamine and histidine decreased with increasing glycemia, reflecting, at least in part, insulin resistance. Only one single nucleotide polymorphism regulating hyperglycemia was significantly associated with amino acid levels. ***Diabetes*** **61:1895–1902, 2012**

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Received 5 October 2011 and accepted 4 March 2012.

DOI: 10.2337/db11-1378

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db11-1378/-/DC1.

Insulin regulates carbohydrate, lipid, protein, and amino acid metabolism (1). Insulin inhibits proteolysis and associated release of amino acids and stimulates amino acid uptake and protein synthesis in skeletal muscle (2,3). Selected amino acids, however, enhance insulin secretion (4,5) or modulate insulin sensitivity (6–10), the two main mechanisms in the regulation of glucose homeostasis.

A recent study reported that three branched-chain amino acids (BCAAs), valine, leucine and isoleucine, and two aromatic amino acids, phenylalanine and tyrosine, predicted type 2 diabetes (11). The risk of diabetes was fivefold higher in individuals in the top quartile of a combination of three amino acids (isoleucine, phenylalanine, and tyrosine) compared with individuals in the lowest quartile. Although some small studies have reported that the levels of amino acids differ between individuals with normal and abnormal glucose tolerance (12,13), previous studies have not investigated the levels of amino acids across the entire range of glucose tolerance.

Amino acids modulate insulin action on glucose transport (9,14–16) and gluconeogenesis (17). High levels of BCAAs, especially leucine, have been shown to associate with insulin resistance (16,18,19) or insulin-resistant states, including diabetes (6,12,13). BCAAs have been shown to downregulate insulin action on glucose uptake by inhibiting critical steps in the postreceptor insulin signaling cascade (10), although other studies have concluded that leucine and isoleucine stimulate glucose uptake (7,8,20). Gluconeogenic amino acids (mainly alanine and glutamine) can enhance hepatic glucose production and thus lead to hyperglycemia (21,22). Finally, amino acids such as arginine, glutamine, leucine, and phenylalanine directly stimulate insulin secretion (4).

Type 2 diabetes is a complex metabolic disease with a significant genetic component, and >40 gene loci associated with the risk of type 2 diabetes or hyperglycemia have been identified (23–26). The mechanisms by which these loci contribute to the risk of diabetes are only partially known. There are no previous studies on the association of these gene variants with the levels of amino acids.

The aims of our study were *1*) to investigate the relationship between the levels of amino acids and fasting and 2-h glucose across the entire range of glucose tolerance, *2*) to investigate the role of insulin sensitivity and insulin secretion in this relationship, *3*) to investigate the relationship between insulin sensitivity and adipose tissue

mRNA expression of genes implicated in the catabolism of amino acids, and *4*) to investigate whether any of 43 risk single nucleotide polymorphisms (SNPs) for type 2 diabetes or hyperglycemia affect serum amino acid levels.

RESEARCH DESIGN AND METHODS

Subjects and clinical measurements. The study included 9,369 nondiabetic or newly diagnosed type 2 diabetic men from the population-based Metabolic Syndrome in Men (METSIM) Study (mean ± SD age, 57 ± 7 years; BMI, 27.0 ± 4.0 kg/m^2). The study design has been described in detail elsewhere (27).

Glucose tolerance was evaluated according to the American Diabetes Association criteria (28). A total of 3,026 subjects (32.3%) had normal glucose tolerance (NGT), 4,327 (46.2%) had isolated impaired fasting glucose (IFG), 312 (3.3%) had isolated impaired glucose tolerance (IGT), 1,058 (11.3%) had IFG and IGT, and 646 (6.9%) had newly diagnosed type 2 diabetes. Additional analyses were performed in 1,775 nondiabetic subjects re-examined during an ongoing follow-up METSIM study, of which 375 maintained NGT, 1,249 remained nondiabetic, and 151 developed new type 2 diabetes during the mean follow-up of 4.7 ± 1.0 years. None of 9,369 subjects was receiving antidiabetic treatment. BMI was calculated as weight (kg) divided by height (m) squared. The study was approved by the ethics committee of the University of Kuopio and Kuopio University Hospital and conducted in accordance with the Helsinki Declaration.

Amino acid measurements. A high-throughput serum nuclear magnetic resonance (NMR) platform operating at 500 MHz was used for amino acid quantification (29). Fasting serum samples collected at the baseline study were stored at −80°C and thawed overnight in a refrigerator before sample preparation. Aliquots of each sample (300 μL) were mixed with sodium phosphate buffer (300 μL). A proton NMR spectrum was acquired where most spectral signals from the macromolecules and lipoprotein lipids were suppressed to enhance detection of the amino acid signals. The eight amino acids (alanine, phenylalanine, valine, leucine, isoleucine, tyrosine, histidine, glutamine) were quantified in standardized concentration units. Details of the NMR experimentation and amino acid quantification have been described previously (29,30).

Insulin sensitivity and insulin secretion indices. Results of oral glucose tolerance testing (OGTT) were used to calculate the Matsuda index of insulin sensitivity (ISI) as 10,000/$\sqrt{}$ (fasting insulin × fasting glucose × mean insulin during OGTT × mean glucose during OGTT) (31). An index of early-phase insulin secretion during an OGTT, insulin area under the curve (InsAUC)$_{0–30}$/glucose (Glu)AUC$_{0–30}$, was calculated as (fasting insulin + 30-min insulin)/(fasting glucose + 30-min glucose) (pmol/mmol) (27).

Genotyping. Genotyping of 43 SNPs (29 SNPs associated with risk for type 2 diabetes and 14 associated with increased fasting or 2-h glucose in an OGTT) (23–26) was performed using the Applied Biosystems TaqMan Allelic Discrimination Assay at the University of Eastern Finland or the Sequenom iPlex Gold SBE assay at the National Human Genome Research Institute at the National Institutes of Health. The TaqMan genotyping call rate was 100%, and the discordance rate was 0% among 4.5% DNA samples genotyped in duplicate. The Sequenom iPlex call rate was 90.2–96.9%, and the discordance rate was 0% among 4.2% DNA samples genotyped in duplicate. All SNPs were in Hardy-Weinberg equilibrium at the significance level corrected for multiple testing by Bonferroni method ($P < 0.0012$). Descriptive data for individual SNPs are shown in Supplementary Table 1.

Gene expression analysis. Total RNA was isolated from 200 subcutaneous fat biopsy samples of METSIM participants using Qiagen miRNeasy kit according to manufacturer's instructions. RNA integrity number values were assessed with the Agilent Bioanalyzer 2100. High-quality samples (RNA integrity number >7.0) were used for transcriptional profiling with the Illumina Human HT-12 v3 Expression BeadChip. Genome Studio software (2010.v3) was used for obtaining fluorescent intensities. The HT-12 BeadChip contains 48,804 expression and 786 control probes. Expression data from 19,306 probes were removed because of *1*) failure of the probe to align to a genomic or transcriptomic location; *2*) alignment of the probe to multiple genomic or transcriptomic locations; or *3*) presence of SNPs in the probe sequence that may affect hybridization efficiency using the methodology developed by Barbosa-Morais et al. (32). The remaining 29,497 probes were processed using nonparametric background correction, followed by quantile normalization with control and expression probes using the *neqc* function in the *limma* package (R v2.13.0) (33). The 16,223 probes with detection P values <0.01 in any of the 200 samples were used for further analysis. Gene expression data have been deposited to Gene Expression Omnibus (GEO) with the accession number GSE32512.

Statistical analysis. Statistical analyses were conducted using SPSS 17 software (SPSS, Chicago, IL). All amino acids, BMI, Matsuda ISI, and early-phase insulin secretion index (InsAUC$_{0–30}$/GluAUC$_{0–30}$) were log-transformed to correct for their skewed distribution. Amino acids were compared across the fasting and 2-h glucose categories using the general linear model adjusted for age and BMI, or additionally for Matsuda ISI or InsAUC$_{0–30}$/GluAUC$_{0–30}$ indices. $P < 0.003$ (corrected for 16 tests by Bonferroni method) was considered statistically significant. Associations between amino acid levels and indices of insulin sensitivity and insulin secretion were evaluated with Pearson correlation coefficients. The association of amino acid levels with newly developed type 2 diabetes was tested with logistic regression adjusted for confounding factors. Correlations between gene expression levels and phenotypes were calculated using the Pearson correlation coefficient. We used the Benjamini–Hochberg false discovery rate (FDR) method (34) to correct for multiple comparisons, and considered an FDR-adjusted $P_{FDR} < 0.05$ statistically significant.

For genetic association analysis, unstandardized effect sizes (B [SE]) per copy of the minor allele were estimated by linear regression analysis adjusted for age and BMI, using untransformed dependent variables, and percentages of B from the mean were calculated. P values were calculated using logarithmically transformed variables when appropriate. A $P < 1.45 \times 10^{-4}$ adjusted for multiple comparisons by Bonferroni method was considered to be statistically significant given a total of 344 tests performed (8 traits × 43 SNPs). We had ≥80% power to detect changes in the mean trait value from 1.4 to 5.9% per copy of the minor allele at the significance level of 0.05, depending on the minor allele frequency (Supplementary Fig. 1). Hardy-Weinberg equilibrium was evaluated by χ^2 test.

RESULTS

Hyperglycemia and the levels of eight amino acids. We generated categories of fasting (FPG) and 2-h (2hPG) plasma glucose (by 0.5 and 1.0 mmol/L steps, respectively) to investigate the relationship between amino acid levels and glycemia in participants with normoglycemia, IFG, IGT, and type 2 diabetes. Categories with FPG <5.0 mmol/L and 2hPG <5.0 mmol/L were set as the reference categories. Across the FPG categories, we observed a significant ($P < 0.003$) increase in isoleucine level of 38% in the highest glucose category versus the reference category ($P = 6.7 \times 10^{-10}$ adjusted for age and BMI), tyrosine (+21%, $P = 1.8 \times 10^{-15}$), alanine (+17%, $P = 4.3 \times 10^{-42}$), phenylalanine (+15%, $P = 1.1 \times 10^{-9}$), and leucine (+19%, $P = 4.2 \times 10^{-4}$) levels, and a significant decrease in histidine (−9%, $P = 3.3 \times 10^{-4}$) and glutamine (−22%, $P = 1.9 \times 10^{-48}$; Fig. 1*A*). Similar trends were seen across the 2hPG categories, with a significant increase in isoleucine (+38%, $P = 2.7 \times 10^{-58}$), tyrosine (+30%, $P = 1.1 \times 10^{-10}$), alanine (+14%, $P = 2.3 \times 10^{-46}$), phenylalanine (+14%, $P = 1.1 \times 10^{-13}$), and leucine (+15%, $P = 1.0 \times 10^{-18}$) levels, and a significant decrease in histidine (−5%, $P = 9.1 \times 10^{-6}$) and glutamine (−12%, $P = 1.4 \times 10^{-31}$) levels with higher 2hPG (Fig. 1*B*). These effects were more significant in obese (BMI ≥27 kg/m^2) than in nonobese (BMI<27 kg/m^2) participants, although the trends were similar in both groups (Supplementary Fig. 2). Overall, most amino acids increased, whereas glutamine and histidine decreased with higher FPG and 2hPG.

Table 1 shows that all amino acids that were increased in hyperglycemia negatively correlated with Matsuda ISI ($r \leq -0.3$, $P \leq 1 \times 10^{-154}$) and positively ($r \geq 0.2$, $P \leq 3 \times 10^{-85}$) with the InsAUC$_{0–30}$/GluAUC$_{0–30}$ index of early-phase insulin secretion. For glutamine, which was decreased in hyperglycemia, the correlations were weaker and in the opposite direction ($r = 0.17$ for Matsuda ISI and −0.12 for InsAUC$_{0–30}$/GluAUC$_{0–30}$). Correlations of amino acid levels (except for histidine) with InsAUC$_{0–30}$/GluAUC$_{0–30}$ were weaker than correlations with Matsuda ISI and were largely attenuated after the adjustment of InsAUC$_{0–30}$/GluAUC$_{0–30}$ for Matsuda ISI. Furthermore, additional adjustment for Matsuda ISI attenuated or abolished most of the associations between glucose categories and amino acid levels (with the exception of valine and histidine), whereas adjustment for InsAUC$_{0–30}$/GluAUC$_{0–30}$ attenuated only the associations for histidine (Supplementary Table 2). Thus, insulin sensitivity seemed to at least partly explain the relationship between glucose and amino acid levels.

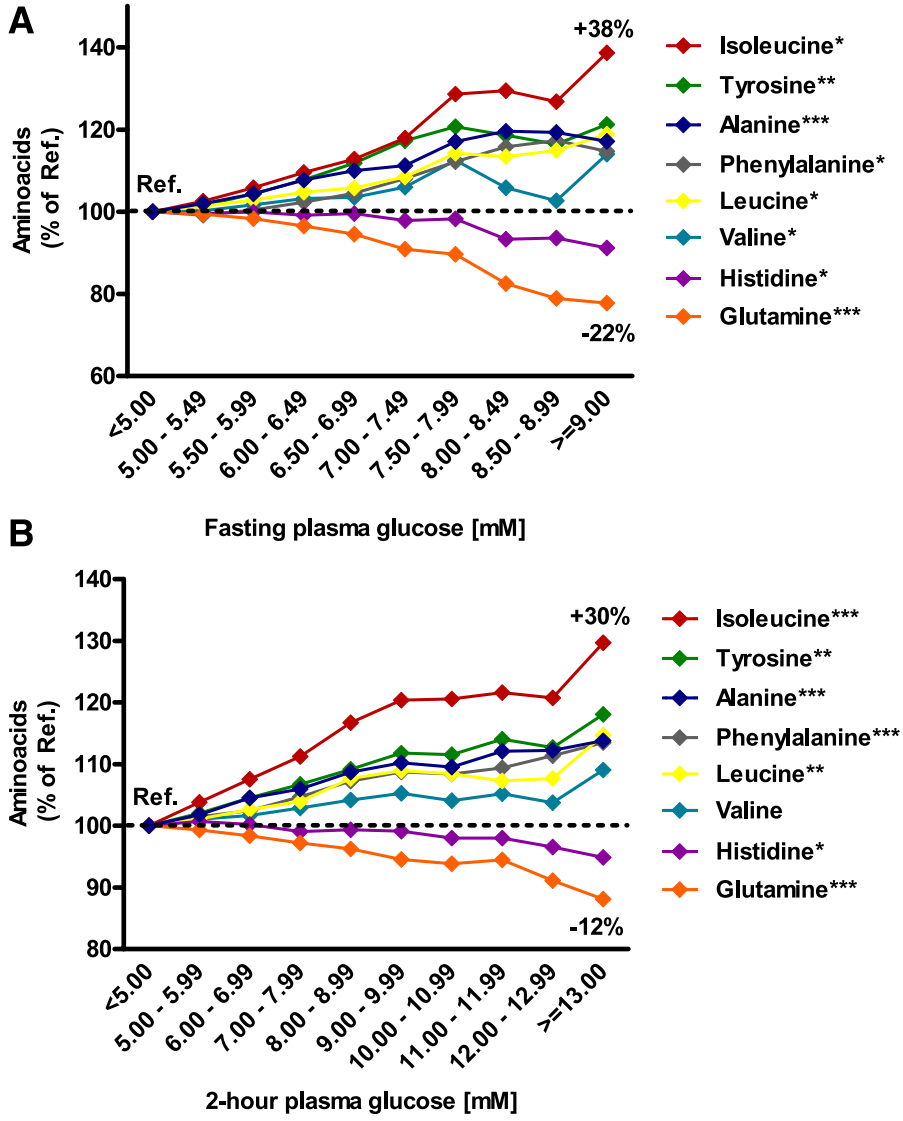

FIG. 1. Amino acid levels in categories of FPG (*A*) and 2hPG (*B*) levels across the entire range of glucose tolerance. Points represent unadjusted means of amino acid levels in each glucose category. *P* values were calculated using general linear model adjusted for age and BMI. *$P < 0.05$. **$P < 10^{-10}$. ***$P < 10^{-30}$.

Association of eight amino acid levels with risk of type 2 diabetes. We investigated the relationship between amino acid levels and the development of type 2 diabetes during a 4.7-year follow-up, including 526 re-examined METSIM participants (375 with NGT at baseline and follow-up examinations, and 151 with type 2 diabetes). Of eight amino acids measured at baseline, high levels of alanine ($P = 6.7 \times 10^{-5}$), leucine ($P = 0.005$), isoleucine

TABLE 1
Pearson correlations between the levels of eight amino acids and indices of insulin sensitivity (Matsuda ISI) and early-phase insulin secretion ($InsAUC_{0–30}/GluAUC_{0–30}$) in nondiabetic METSIM participants

	Matsuda ISI			$InsAUC_{0–30}/GluAUC_{0–30}$			$InsAUC_{0–30}/GluAUC_{0–30}$ adjusted for ISI		
	N	*r*	*P*	*N*	*r*	*P*	*N*	*r*	*P*
Alanine	8,672	−0.379	1E-294	8,678	0.273	2E-148	7,828	−0.029	0.010
Phenylalanine	8,661	−0.340	1E-232	8,667	0.256	2E-129	7,828	−0.006	0.609
Leucine	8,661	−0.279	1E-154	8,667	0.208	3E-85	7,828	−0.019	0.096
Isoleucine	8,666	−0.485	<1E-294	8,672	0.388	<2E-148	7,828	0.027	0.017
Tyrosine	8,582	−0.400	<1E-294	8,588	0.293	4E-170	7,828	−0.025	0.025
Valine	8,661	−0.296	2E-174	8,667	0.229	6E-104	7,828	−0.001	0.908
Histidine	8,672	−0.024	0.026	8,678	0.045	3E-05	7,828	0.042	9E-05
Glutamine	7,871	0.172	2E-53	7,876	−0.096	1E-17	7,828	0.054	1E-06

($P = 3.3 \times 10^{-5}$), tyrosine ($P = 0.001$), and phenylalanine ($P = 0.048$) were significantly associated with incident diabetes (adjusted for age and BMI; Table 2). Additional adjustment for Matsuda ISI alone, or Matsuda ISI and $InsAUC_{0–30}/GluAUC_{0–30}$, but not for $InsAUC_{0–30}/GluAUC_{0–30}$ alone, abolished statistical significances, suggesting that the association of these amino acids with incident diabetes was mostly explained by insulin resistance. Further adjustment for fasting hyperglycemia did not essentially change the association, but adjustment for 2-h glucose made the association for alanine statistically significant ($P = 0.027$). The elevated baseline glutamine level was significantly associated with a decreased risk of newly developed type 2 diabetes ($P = 4.1 \times 10^{-6}$), and this association persisted after the adjustment for Matsuda ISI ($P = 1.5 \times 10^{-4}$) or $InsAUC_{0–30}/GluAUC_{0–30}$ ($P = 6.7 \times 10^{-6}$) or both ($P = 0.048$). Additional adjustment for fasting glucose abolished this association ($P = 0.051$). The levels of glutamine were significantly lower at baseline in 440 participants who developed abnormal glucose tolerance (IGT and/or IFG, diabetes) at follow-up, compared with 375 participants who remained normoglycemic (155.6 vs. 159.6; $P = 0.019$, adjusted for age and BMI; Supplementary Table 3). We also performed statistical analyses in a larger sample, including all 1,624 reexamined participants who did not develop diabetes during the follow-up and 151 participants who developed type 2 diabetes. The results remained essentially similar, although P values were less significant.

Gene expression of genes involved in amino acid metabolism in relation to insulin sensitivity. Analysis of microarray data from subcutaneous adipose tissue samples of 200 METSIM participants showed that Matsuda ISI correlated significantly with mRNA levels of several genes involved in the metabolism of alanine, including a key enzyme, alanine aminotransferase ($r = 0.46$, $P_{FDR} = 9.7 \times 10^{-9}$); glutamine, including a key enzyme, glutamine synthetase ($r = 0.44$, $P_{FDR} = 4.0 \times 10^{-8}$); BCAA, including key enzymes of BCAA degradation, branched chain amino-acid transaminase 2 ($r = 0.36$, $P_{FDR} = 2.5 \times 10^{-5}$) and branched-chain α-keto acid dehydrogenase (BCKDH) A and B ($r = 0.45$ and 0.35, $P_{FDR} = 1.3 \times 10^{-8}$ and 3.0×10^{-5}); and phenylalanine, tyrosine, and histidine (Supplementary Table 4). The most consistent results were observed for the BCAA degradation, as enzyme mRNAs correlated positively with Matsuda ISI at almost all steps of the metabolic pathway (Supplementary Fig. 3). Figure 2 shows that the mRNA levels of three key enzymes in BCAA degradation also correlated negatively with ISI, FPG, and 2hFP levels. However, these associations disappeared when controlled for Matsuda ISI. These results indicate that the association between insulin sensitivity and amino acid catabolism (especially BCAA catabolism) also exists at the mRNA level and add further evidence that insulin sensitivity is likely contributing to higher BCAA levels in hyperglycemia.

Risk variants for type 2 diabetes and/or hyperglycemia and the levels of eight amino acids. Of the 43 SNPs investigated, only *GCKR* rs780094 was significantly associated with the levels of several amino acids after Bonferroni correction for multiple testing ($P < 1.45 \times 10^{-4}$) (Fig. 3 and Supplementary Table 1). The glucose-increasing C (major) allele was associated with lower levels of alanine (effect size −1.9% per C allele, $P = 1.6 \times 10^{-11}$) and isoleucine (−2.3%, $P = 3.1 \times 10^{-6}$), and higher levels of glutamine (+1.2%, $P = 1.0 \times 10^{-6}$ adjusted for age and BMI). The C allele also had nominally significant effects on leucine (−1.2%, $P = 0.001$), tyrosine (+1.1%, $P = 0.001$), and histidine (+0.8%, $P = 0.003$). These associations did not change significantly after additional adjustment for FPG, 2hPG, or Matsuda ISI (data not shown), traits known to be modulated by SNPs of *GCKR*. Furthermore, the association of amino acids with newly developed type 2 diabetes was not affected by further adjustment for rs780094.

A number of nominally significant associations of other SNPs with amino acids were found; the top ranking was rs8042680 (*PRC1*). The type 2 diabetes risk allele (A) was nominally associated with higher levels of leucine (+1.2%, $P = 0.002$), isoleucine (+1.7%, $P = 0.001$), and valine (+1.2%, $P = 0.001$; Supplementary Table 1). The most consistent nominally significant associations were seen for rs75789326 (*IRS1*). The nonrisk allele (G) was associated with lower leucine (−0.8%, $P = 0.019$), isoleucine (−1.5%, $P = 0.003$), tyrosine (−0.7%, $P = 0.037$), valine (−0.7%, $P = 0.047$), and histidine (−0.5%, $P = 0.039$) levels.

DISCUSSION

This is the first large, population-based study aiming to investigate the relationship between hyperglycemia and 43 risk SNPs for type 2 diabetes/hyperglycemia and amino acid levels. We observed that with increasing FPG and/or 2hPG, the levels of alanine, valine, leucine, isoleucine, phenylalanine, and tyrosine increased, whereas the levels of histidine and glutamine decreased. Significant correlations between

TABLE 2
Association between the baseline levels of eight amino acids and newly developed type 2 diabetes during the follow-up of the METSIM study participants (logistic regression adjusted for age, BMI, and additional covariates)

	Odds ratio (95% CI)	P*	P†	P‡	P§	P‖	P¶
Alanine	1.024 (1.012–1.037)	**6.7E-05**	0.154	**6.0E-07**	0.895	0.191	**0.027**
Glutamine	0.973 (0.962–0.985)	**4.1E-06**	**1.5E-04**	**6.7E-06**	**0.048**	0.051	**0.007**
Histidine	0.958 (0.885–1.038)	0.296	0.132	0.469	0.187	0.794	0.127
Isoleucine	1.101 (1.052–1.152)	**3.3E-05**	0.293	**5.2E-08**	0.649	**0.013**	0.064
Leucine	1.047 (1.014–1.081)	**0.005**	0.246	**0.002**	0.379	0.253	0.197
Phenylalanine	1.062 (1.001–1.128)	**0.048**	0.879	**0.003**	0.292	0.146	0.630
Tyrosine	1.118 (1.048–1.193)	**0.001**	0.466	**1.2E-05**	0.528	0.142	0.139
Valine	1.013 (0.995–1.031)	0.149	0.657	**0.050**	0.264	0.408	0.940

Subjects with newly diagnosed type 2 diabetes at follow-up ($N = 151$) were compared against those with NGT/NFG at the baseline and follow-up examinations ($n = 375$). Bold type indicates statistical significance. *Adjustment for age and BMI. †Adjustment for age, BMI, and Matsuda ISI. ‡Adjustment for age, BMI, and $InsAUC_{0–30}/GluAUC_{0–30}$. §Adjustment for age, BMI, $InsAUC_{0–30}/GluAUC_{0–30}$, and Matsuda ISI. ‖Adjustment for age, BMI, $InsAUC_{0–30}/GluAUC_{0–30}$, Matsuda ISI, and FPG. ¶Adjustment for age, BMI, $InsAUC_{0–30}/GluAUC_{0–30}$, Matsuda ISI, and 2hPG.

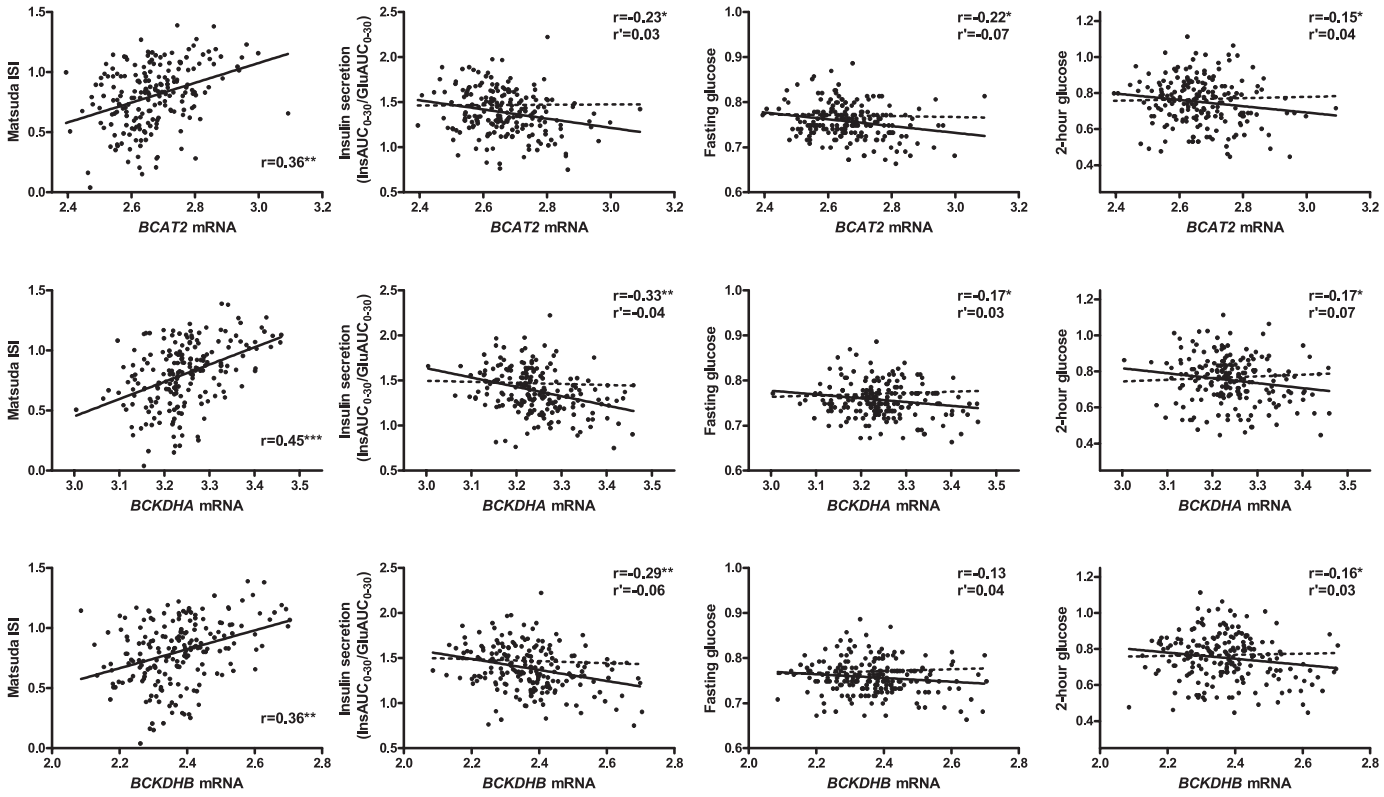

FIG. 2. Correlations of mRNA levels of three key enzymes in BCAA degradation with insulin sensitivity (Matsuda ISI), early-phase insulin secretion ($InsAUC_{0–30}/GluAUC_{0–30}$), FPG and 2hPG. *r'*, partial Pearson correlation coefficient controlled for Matsuda ISI. *$P < 0.05$. **$P < 1 \times 10^{-4}$. ***$P < 1 \times 10^{-10}$. The solid line indicates the linear regression line for the unadjusted variable, and the dotted line indicates linear regression line for the variable adjusted for Matsuda ISI.

insulin sensitivity and mRNA expression of genes regulating amino acid metabolism were found. Only 1 SNP (rs780094 in *GCKR*) of 43 risk SNPs for type 2 diabetes or hyperglycemia was significantly associated with the levels of several amino acids.

Hyperglycemia and levels of eight amino acids. We demonstrated that the levels of alanine, phenylalanine, valine, leucine, isoleucine, and tyrosine increased and the levels of histidine and glutamine decreased in hyperglycemia, indicating that the changes in amino acid levels parallel closely the changes in FPG and 2hPG levels. Similar trends were also observed in normoglycemia, suggesting that even mild elevations of glucose levels result in changes in amino acid levels. The largest and most significant increase across the FPG and 2hPG categories was observed for isoleucine (increased up to 38 and 30%, respectively, compared with the reference category), and alanine (increased up to 17 and 14%), and the largest decrease was seen for glutamine (by 22 and 12%, respectively). We also showed that obesity did not have a major effect on these associations, although the effects of hyperglycemia on the levels of amino acids tended to be more pronounced in obese individuals.

The levels of all amino acids correlated inversely (except for glutamine correlating positively) with insulin sensitivity (Matsuda ISI). The strongest correlation was found for isoleucine ($r = -0.49$), a BCAA previously shown to be a potent activator of glucose uptake in skeletal muscle (7,8). Insulin resistance is likely to be an important mechanism explaining these associations because the adjustment for Matsuda ISI attenuated or abolished most of the associations between glucose (especially 2hPG) and amino acid levels. Further evidence for the role of insulin resistance mediating the associations of amino acids with hyperglycemia comes from our 4.7-year prospective follow-up of the METSIM cohort. We demonstrated that alanine, leucine, isoleucine, tyrosine, and glutamine were associated with incident type 2 diabetes. Adjustment for insulin sensitivity abolished significant associations, with the exception of glutamine, indicating that insulin resistance is likely to play an important role in the risk of type 2 diabetes induced by amino acids. Insulin secretion or hyperglycemia did not significantly modify these associations. Our study not only supports the recent observations of a relationship between amino acid levels with the risk of diabetes (11) but also offers a possible underlying mechanism to explain this relationship.

Our study found that the levels of BCAAs (particularly isoleucine) were strongly correlated with mRNA levels of genes involved in BCAA catabolism. BCAAs are thought to be early indicators of insulin resistance (6,10,18,35). High levels of BCAAs promote insulin resistance by interfering with the insulin-signaling pathway (10) or by directly inhibiting muscle glucose transport and/or phosphorylation in skeletal muscle (9), whereas insulin resistance in turn may contribute to high levels of amino acids by increased release of BCAAs (and other amino acids) due to impaired ability of insulin to suppress proteolysis in skeletal muscle (36). BCAAs are oxidized in skeletal muscle and adipose tissue (37) to form alanine and glutamine (38). Oxidation of BCAAs was increased in individuals with type 2 diabetes (21). In animal models of type 2 diabetes, BCAA catabolism is downregulated due to low activity of BCKDH complex (39), the key enzyme of BCAA oxidation. In our study, adipose tissue mRNA expression of *BCKDHA* and

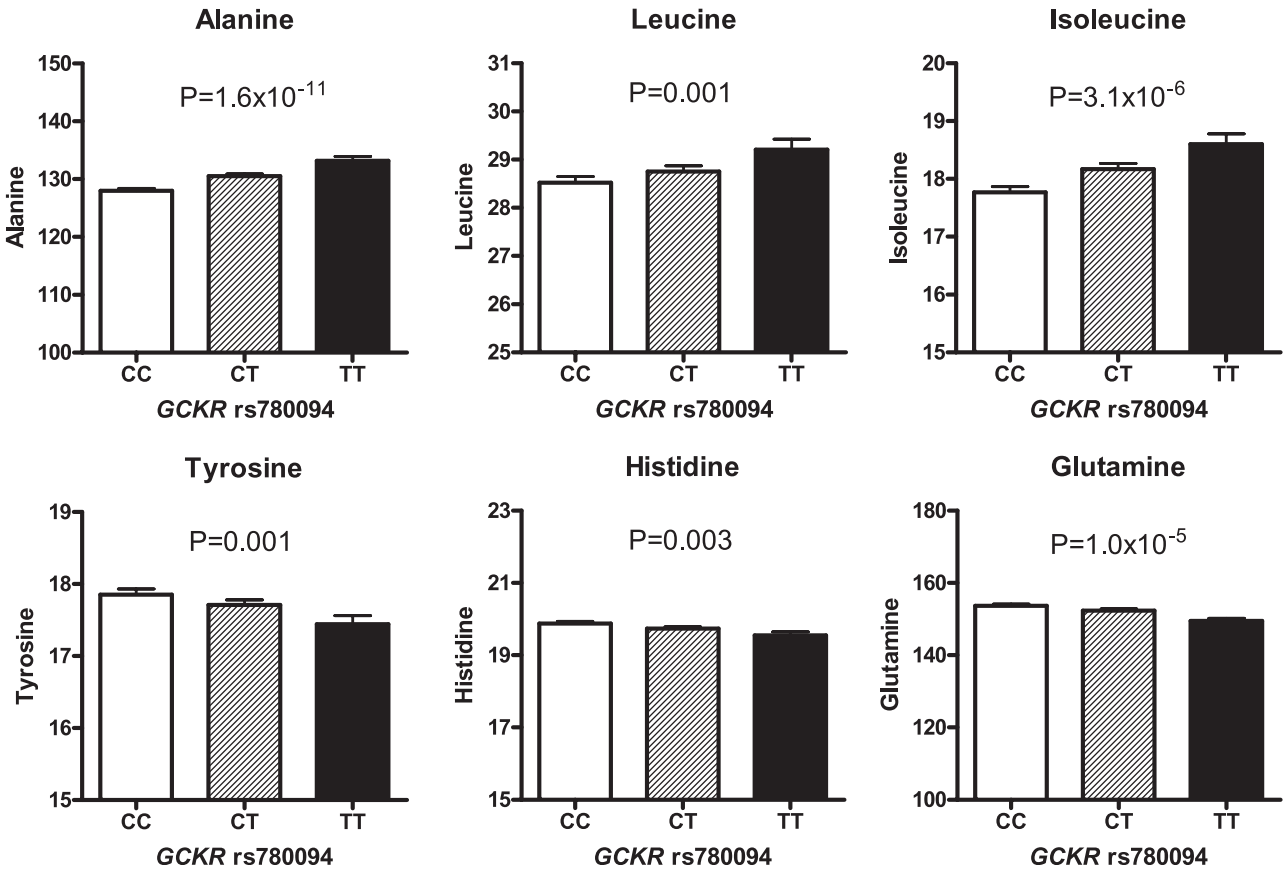

FIG. 3. Associations between *GCKR* rs780094 and amino acid levels in nondiabetic men. Bars show unadjusted means ± SE. *P* values were adjusted for age and BMI with linear regression. CC, homozygotes for the glucose-increasing major allele; CT, heterozygotes; TT, homozygotes for the glucose-decreasing minor allele.

BCKDHB (encoding α and β polypeptides of BCKDH E1) as well as of *BCAT2* (branched chain amino-acid transaminase 2 mitochondrial, the first enzyme in BCAA catabolism) and other genes involved in BCAA catabolism correlated positively with Matsuda ISI and negatively with glucose levels. This suggests that the insulin resistance-related decrease in degradation of BCAAs could be one of the mechanisms leading to the elevation of BCAA levels in hyperglycemia in humans. Other possible mechanisms for the elevation of BCAAs in hyperglycemia could be a decreased uptake and increased release of BCAAs from skeletal muscle due to increased protein catabolism in insulin resistance.

Alanine is a nonessential amino acid synthesized from pyruvate and amino acids (mainly BCAAs) primarily in skeletal muscle and gut and used for gluconeogenesis in the liver. Alanine is the main precursor for gluconeogenesis in the liver (38,40) and a stimulator of glucagon secretion (41). Therefore, high plasma levels of alanine may contribute especially to fasting hyperglycemia by enhancing gluconeogenesis. Our study shows that elevated levels of alanine in hyperglycemia, both in obese and nonobese individuals, could be mediated by insulin resistance, which is supported by a significant positive correlation between insulin sensitivity and mRNA level of alanine aminotransferase, the key enzyme in alanine metabolism.

Glutamine is a nonessential gluconeogenic amino acid synthesized from glucose or amino acids or released by proteolysis from skeletal muscle (42). Unlike alanine, glutamine is primarily taken up by the gut and kidneys, where it is processed to glucose via the gluconeogenesis pathway. Glucagon, but not insulin sensitivity, seems to play a role in the uptake of glutamine by the gut (43). Differential regulation of glutamine metabolism, compared with other amino acids, could possibly explain the opposite associations between glutamine and glucose metabolism. We found that plasma levels of glutamine decreased with elevated levels of FPG and 2hPG and that a low level of glutamine was significantly associated with future diabetes, independently of obesity and insulin resistance. Baseline glutamine levels were already decreased in normoglycemic participants who developed abnormal glucose tolerance at follow-up compared with those who remained normoglycemic. Our results are in agreement with previous studies showing that patients with type 2 diabetes (12) and lean insulin-resistant normoglycemic offspring of patients with type 2 diabetes have lower levels of glutamine than normoglycemic individuals (44).

Risk variants for type 2 diabetes and/or hyperglycemia and the levels of eight amino acids. A common variant in *GCKR* was the only SNP among 43 loci associated with the levels of several amino acids. *GCKR* encodes the glucokinase regulatory protein, which inhibits the effects of glucokinase (GCK) on glycogen synthesis and glycolysis in the liver (45). SNPs at the *GCKR* locus have been associated with fasting glycemia (24,46), risk of type 2 diabetes (46,47), insulin resistance (46–48), and elevated hepatic glucose uptake (49). In our study, the glucose-increasing major C allele of the intronic SNP rs780094 was significantly and unexpectedly associated with decreased levels of alanine and isoleucine and elevated levels of glutamine.

Mechanisms explaining the relationship of rs780094 of *GCKR* with amino acids are unknown, but the association with low levels of alanine could be a consequence of reduced glycolysis induced by *GCKR* resulting in decreased production of pyruvate and its conversion to alanine. On the basis of our study, it remains unclear whether the effects of rs780094 of *GCKR* on amino acid metabolism are primary or secondary. Although in our study the C allele of rs780094 was associated with both FPG and 2hPG, as well as with Matsuda ISI, the association of *GCKR* with the levels of amino acids was independent of these variables (data not shown). The *GCKR* variant did not affect the association of amino acids with newly developed type 2 diabetes, which agrees with its modest effect on amino acid levels.

Although several type 2 diabetes/hyperglycemia risk SNPs showed nominally significant associations with the levels of selected amino acids, the most consistent results were found for rs7578326 near *IRS1*. The major A allele previously linked to type 2 diabetes risk in a genome-wide association study (26) was nominally associated with elevated levels of several amino acids (valine, leucine, isoleucine, tyrosine, histidine). *IRS1* encodes the insulin receptor substrate 1, an important component of the insulin-signaling pathway, and its gene variant affects insulin resistance and adiposity (50). The association of *IRS1* with amino acid levels, if confirmed, could contribute to evidence for a causal relationship between insulin resistance and amino acid metabolism. The type 2 diabetes risk allele of the rs8042680 in *PRC1*, encoding protein regulator of cytokinesis 1, was associated specifically but nominally with higher levels of all three BCAAs. *PRC1* is not known to have a function in amino acid metabolism. These results suggest that the relationship between the levels of glucose and amino acids could be determined, at least in part, by genes regulating hyperglycemia or the risk of diabetes.

This study has limitations. Only Finnish men were included, and therefore, we do not know whether our results are applicable to women and to different ethnic or racial groups. We had only a modest statistical power to demonstrate statistically significant associations of gene variants with amino acids.

In conclusion, our large, population-based study shows that levels of branched-chain, aromatic amino acids and alanine increase whereas glutamine and histidine decrease with increasing FPG and/or 2hPG levels. These associations seemed to be mediated by insulin resistance, at least in part, supported by correlations of expression of genes involved in amino acid metabolism with the Matsuda ISI. Among the 43 loci associated with risk for hyperglycemia or type 2 diabetes, only *GCKR* rs780094 was significantly associated with several amino acids, especially with the levels of alanine.

ACKNOWLEDGMENTS

This work was supported by the Academy of Finland (A.S., M.L., SALVE program to M.A.-K.), the Finnish Diabetes Research Foundation (M.L.), the Finnish Cardiovascular Research Foundation (M.L., M.A.-K.), the Jenny and Antti Wihuri Foundation (A.J.K.), an EVO grant from the Kuopio University Hospital (5263), DK062370 to M.B., 1Z01 HG000024 to F.S.C., and National Institutes of Health Grant HL-095056 to P.P. and Grant HL-28481 to P.P. and A.J.L.

No potential conflicts of interest relevant to this article were reported.

A.S. wrote the manuscript and researched the data. M.C., N.K.S., P.P., and A.J.L. performed the mRNA experiments, analyzed the data, and reviewed and edited the manuscript. P.S. conceived, designed, and performed the NMR experiments, analyzed the data, and reviewed and edited the manuscript. A.J.K. analyzed the NMR data, contributed analysis tools, and reviewed and edited the manuscript. H.C., J.Pa., and J.Pi. researched the data and reviewed and edited the manuscript. L.L.B., M.A.M., and F.S.C. designed and performed genotyping and reviewed and edited the manuscript. M.B. contributed analysis tools and reviewed and edited the manuscript. J.K. designed the study and reviewed the manuscript. M.A.-K. conceived and designed the NMR experiments, analyzed the data, and reviewed and edited the manuscript. M.L. designed the study, contributed to discussion, and reviewed and edited the manuscript. M.L. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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