α-Hydroxybutyric Acid Is a Selective Metabolite Biomarker of Impaired Glucose Tolerance

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OBJECTIVE

Plasma metabolites that distinguish isolated impaired glucose tolerance (iIGT) from isolated impaired fasting glucose (iIFG) may be useful biomarkers to predict IGT, a high-risk state for the development of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Targeted metabolomics with 23 metabolites previously associated with dysglycemia was performed with fasting plasma samples from subjects without diabetes at time 0 of an oral glucose tolerance test (OGTT) in two observational cohorts: RISC (Relationship Between Insulin Sensitivity and Cardiovascular Disease) and DMVhi (Diabetes Mellitus and Vascular Health Initiative). Odds ratios (ORs) for a one-SD change in the metabolite level were calculated using multiple logistic regression models controlling for age, sex, and BMI to test for associations with iIGT or iIFG versus normal. Selective biomarkers of iIGT were further validated in the Botnia study.

RESULTS

α-Hydroxybutyric acid (α-HB) was most strongly associated with iIGT in RISC (OR 2.54 [95% CI 1.86–3.48], *P* value 5E-9) and DMVhi (2.75 [1.81–4.19], 4E-5) while having no significant association with iIFG. In Botnia, α-HB was selectively associated with iIGT (2.03 [1.65–2.49], 3E-11) and had no significant association with iIFG. Linoleoyl-glycerophosphocholine (L-GPC) and oleic acid were also found to be selective biomarkers of iIGT. In multivariate IGT prediction models, addition of α-HB, L-GPC, and oleic acid to age, sex, BMI, and fasting glucose significantly improved area under the curve in all three cohorts.

CONCLUSIONS

α-HB, L-GPC, and oleic acid were shown to be selective biomarkers of iIGT, independent of age, sex, BMI, and fasting glucose, in 4,053 subjects without diabetes from three European cohorts. These biomarkers can be used in predictive models to identify subjects with IGT without performing an OGTT.

There are an estimated 86 million people with prediabetes in the U.S. (1), with an estimated prevalence of isolated impaired glucose tolerance (iIGT) and combination of impaired fasting glucose (IFG) and IGT of 5.4 and 9.8%, respectively, among U.S. adults (2). In addition, there are 1.7 million new cases of type 2 diabetes (T2DM) diagnosed annually in the U.S. (1). Numerous studies have shown that T2DM can be prevented or delayed with lifestyle and pharmacological intervention in at-risk subjects, in particular in subjects with IGT (3,4). There is a need, however, to better

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identify at-risk subjects and to focus prevention efforts on those who will benefit the most. Patients with combined IFG and IGT are among those at highest risk to progress to T2DM (5). IFG is diagnosed from a fasting plasma sample (fasting plasma glucose [FPG]), whereas IGT diagnosis involves the use of the oral glucose tolerance test (OGTT). The OGTT is unpopular with primary care physicians and patients, and its use has been largely supplanted by the more convenient FPG and HbA_{1c} measurements as diagnostic tools (6,7) outside of the setting of gestational diabetes screening (8). Nevertheless, IGT categorization remains a useful tool for diabetes risk assessment; a simple means of identifying subjects with IGT would therefore be valuable.

Targeted and nontargeted metabolomics profiling have expanded our understanding of the metabolic changes that occur during the progression from normal glucose tolerance (NGT) to T2DM (9). A number of metabolite biomarkers that can predict incident diabetes have been identified. These include the branched-chain amino acids (BCAAs) (10), aromatic amino acids (10,11), glycine (12), α-hydroxybutyric acid (α-HB) (13), and linoleoyl-glycerophosphocholine (L-GPC) (11–13). Within the context of prediabetes, a number of metabolites have been shown to be associated with IGT (12) and IFG (14), but there are no reports of metabolites associated with the discrete and nonoverlapping dysglycemic states of isolated IFG (iIFG) and iIGT. These states have partly distinct metabolic profiles and, presumably, might also exhibit distinct metabolite profiles.

The goal of this work was to identify circulating metabolites that differ between iIFG and iIGT and to test whether selective metabolite biomarkers of iIGT can be used as biomarkers of IGT independent of fasting glucose levels. Targeted metabolomics, using a panel of 23 metabolites previously associated with dysglycemia and T2DM (15) in subjects without diabetes from the Relationship Between Insulin Sensitivity and Cardiovascular Disease (RISC) study (16), was used to identify candidate biomarkers of iIGT. Validation of the candidate biomarkers was carried out in another cohort of subjects at risk for T2DM, the Diabetes Mellitus and Vascular Health Initiative (DMVhi) (17). Finally, an additional validation step was performed with the Botnia (18) cohort for the top three biomarkers of iIGT.

RESEARCH DESIGN AND METHODS

Study Cohorts

The RISC study is a prospective, observational cohort study (n = 1,308) whose rationale and methodology have been published (19). In short, clinically healthy participants aged 30–60 years were recruited at 19 centers in 13 countries in Europe. A standard 75-g glucose OGTT was performed at the initial examination. This was followed, within a week, by a hyperinsulinemic-euglycemic clamp (using an insulin infusion rate of 1 mU · min^{-1} · kg^{-1}) for the measurement of insulin sensitivity (whole-body insulin-induced glucose uptake [M_{wb}]). A second OGTT was performed at the 3-year follow-up examination. Fasting blood samples were obtained at each examination, and anthropometric and metabolic parameters were measured at the time of each OGTT. Analyses were carried out on fasting plasma samples from 955 subjects without diabetes taken at time = 0 of an OGTT given at the 3-year follow-up. For the purposes of this study, RISC refers to the 3-year follow-up samples.

The DMVhi screening study recruited 29,458 policy holders from a large Irish private health insurance company, Vhi Healthcare Ireland (17). Between 2009 and 2012, policy holders from two large urban areas aged 45–75 years, with no history of diabetes, were screened for diabetes risk. A subcohort of 700 participants at baseline were identified for the DEXLIFE study (20). This subcohort included participants with IFG (13%) and/or IGT (7%) at baseline, along with normoglycemic participants with an elevated diabetes risk based on FINDRISC (21) at baseline (80%). Analyses were carried out on fasting plasma samples from 668 subjects without diabetes taken at time = 0 of an OGTT given at the 3-year follow-up. For the purposes of this study, DMVhi refers to the 3-year follow-up samples.

The Botnia Study is a family-based, observational study started in 1990 on the west coast of Finland with a goal of identifying diabetes susceptibility genes (18). The prospective part included 2,152 first-degree relatives of patients with T2DM and 528 age- and weight-matched spouses without a family history of T2DM. A standard 75-g, 2-h OGTT was performed at the initial examination and repeated at 2- to 3-year intervals. Fasted blood samples were obtained at each examination, and anthropometric and metabolic parameters were measured at the time of each OGTT. During a median 9.5 years of follow-up, 151 subjects developed T2DM. Analyses were carried out on fasting plasma samples from 2,430 subjects without diabetes taken at time = 0 of an OGTT given at study baseline.

The subjects without diabetes in RISC and Botnia evaluated here do not meet the FPG or OGTT criteria for T2DM (HbA_{1c} was not measured) as per the current American Diabetes Association guidelines. The subjects in DMVhi did not meet FPG, OGTT, or HbA_{1c} criteria for T2DM.

Ethics committee approval was obtained at each recruiting center for each cohort. Subjects provided written informed consent.

Quantitative (Targeted) Assays (23-Metabolite Panel Used in RISC and DMVhi)

For absolute quantitation, metabolites were analyzed by isotope dilution ultra-high-performance liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) assays. In brief, 50 μL of EDTA plasma was spiked with stable labeled internal standards and subsequently subjected to protein precipitation by mixing with 200 μL of 1% formic acid in methanol. After centrifugation, aliquots of clear supernatant were injected onto an Agilent 1290/AB Sciex QTrap 5500 mass spectrometer LC-MS/MS system equipped with a turbo ion-spray source using three different chromatographic systems (mobile phase/column combinations). α-HB, β-hydroxybutyric acid (β-HB), 3-hydroxyisobutyric acid (3-HIB), 3-methyl-2-oxobutyric acid (3-MOB), 3-methyl-2-oxopentanoic acid (3-MOP), 4-methyl-2-oxobutyric acid (4-MOP), L-GPC, and oleic acid were eluted with a gradient (mobile phase A: 0.01% formic acid in water; mobile phase B: acetonitrile/methanol, 1:1) on a Waters Acquity C-18 BEH column (2.1 × 100 mm, 1.7-μm particle size) and detected in negative mode. 2-Aminoadipic acid (2-AAA), creatine, glycine, hydroxyisovaleroyl carnitine, isoleucine, leucine, phenylalanine, serine, trigonelline, tyrosine, and vitamin B5 were eluted with a gradient (mobile phase A: 0.05% perfluoropentanoic acid

in water; mobile phase B: 0.05% perfluoropentanoic acid in acetonitrile) on a Waters Acquity C-18 BEH column (2.1 × 100 mm, 1.7-mm particle size) and detected in positive mode. A 60-μL aliquot of the clear supernatant was diluted with 60 μL of water and injected onto the same column type as above. X-12063 was eluted with a gradient (mobile phase A: 6.5 mmol/L ammonium bicarbonate in water; mobile phase B: 6.5 mmol/L ammonium bicarbonate in methanol/water, 19:1) and detected in negative mode. To an additional 60-μL aliquot of clear supernatant, 60 μL of a 2,4-dinitrophenylhydrazine solution (1% 2,4-dinitrophenylhydrazine in acetonitrile/acetic acid, 85:15) was added. An aliquot of the reaction mixture was injected onto a Waters Acquity/Thermo Quantum Ultra LC-MS/MS system equipped with a heated electrospray ionization source. The 2,4-dinitrohydrazone derivatives of α-ketoglutaric acid and α-ketobutyric acid (α-KB) were eluted with a gradient (mobile phase A: water/ammonium bicarbonate, 500:1; mobile phase B: acetonitrile/methanol, 1:1) on a Waters Acquity C-18 BEH column (2.1 × 50 mm, 1.7-mm particle size) and detected in negative mode.

Quantitation was performed based on the area ratios of analyte and internal standard peaks using a weighted linear least squares regression analysis generated from fortified calibration standards in water, prepared immediately prior to each run. Stable isotope-labeled compounds (α-HB-d_3, β-HB-d_4 [used for both β-HB and 3-HIB], 2-AAA-d_3, 3-MOB-d_7, 4-MOP-d_3 [used for both 3-MOP and 4-MOP], α-KB-$^{13}C_4$, α-ketoglutaric acid-$^{13}C_4$-$^{15}N_2$, creatine-d_3, glycine-$^{13}C_2$-^{15}N, hydroxyisovaleroyl carnitine-d_3, isoleucine-$^{13}C_6$, leucine-d_3, L-GPC-d_9, oleic acid-$^{13}C_{18}$, phenylalanine-d_8, serine-d_3, trigonelline-d_3, tyrosine-d_4, valine-$^{13}C_5$-^{15}N, vitamin B5-$^{13}C_3$-^{15}N, and dehydroepiandrosterone sulfate-d_6 [for X-12063]) were used as internal standards.

Between-run precision was evaluated by analyzing 12 samples of a pooled plasma lot per run at endogenous analyte levels over three separate runs. Between-run % coefficients of variation for the metabolites are as follows: α-HB (5.1), β-HB (4.9), 2-AAA (9.0), 3-HIB (5.3), 3-MOB (7.2), 3-MOP (11.5), 4-MOP (5.7), α-KB (7.0), α-ketoglutaric acid (5.6), creatine (5.0), glycine (5.8), hydroxyisovaleroyl carnitine (5.8), isoleucine (7.1), leucine (5.1), L-GPC (5.0), oleic acid (6.3), phenylalanine (5.1), serine (5.0), trigonelline (5.0), tyrosine (5.1), valine (4.7), vitamin B5 (8.6), and X-12063 (6.3).

Quantitative measurements of α-HB, L-GPC, and oleic acid in Botnia were made as described previously (13).

Statistical Analysis

Metabolite data were rank normalized using the GenABEL package in R to create a normal distribution. Disease states were classified as normal (NGT and normal fasting glucose, FPG <100 and 2-h plasma glucose [2hPG] <140 mg/dL), iIFG (100≤ FPG <126 and 2hPG <140 mg/dL), iIGT (FPG <100 and 140≤ 2hPG <200 mg/dL), and combined IFG and IGT (100≤ FPG <126 and 140≤ 2hPG <200 mg/dL). The associations of metabolites for normal versus iIFG, iIGT, or combined IFG and IGT were made using multiple logistic regression models controlling for age, sex, and BMI. Odds ratios (ORs) for a one-SD change in the metabolite level, 95% CIs, and *P* values (adjusted with a false discovery rate of 0.1) were calculated for each metabolite. ORs were directly compared using their CIs and likelihood ratio tests.

Multiple logistic regression analyses were generated in R (22) to compare the performance of clinical variables and metabolites for their ability to distinguish IGT as a categorical variable in mixed NGT and IGT populations. The performance of each model was assessed as the *c* statistic (the area under the receiver operating characteristic curve [AUC]) generated using the package pROC (23). In addition, within each cohort, the performance of the models was compared with the age/sex/BMI/FPG model by implementing the nonparametric method of DeLong et al. (24). JMP 9.0 (SAS Institute, Cary, NC) was used for additional statistical calculations and *P* values ≤0.05 were considered statistically significant. It is important to note that the *P* values presented are not corrected for multiple comparisons, and although the vast majority of results would hold to even conservative Bonferroni corrections, the *P* values should be interpreted with this in mind.

RESULTS

Quantitative measurements were made for 22 metabolites in fasting plasma samples taken just prior to an OGTT from the RISC and DMVhi cohorts. In addition, relative levels of the unnamed metabolite X-12063 (25) were measured in these two cohorts. X-12063 has a known mass spectrometric signature but its chemical structure has not yet been elucidated. The demographic characteristics of the RISC cohort are shown in Table 1 and those of the DMVhi and Botnia cohorts in Supplementary Table 1, categorized as normal, iIGT, iIFG, and combined IFG and IGT. The 23 metabolites measured in this study are listed along with their abbreviations and classifications in Table 2, which also tallies their median levels in the RISC study for each glycemic category. All 23 metabolites, except for one (α-ketoglutaric acid [α-KG]), were found to be significantly different from normal in at least one prediabetic category in RISC. Similar results (data not shown) were seen in the DMVhi cohort, which also showed a significant change for α-KG in all three dysglycemic states. Most of the metabolites tended to be elevated in dysglycemia versus normal, with the exception of glycine, serine, trigonelline, and L-GPC, which had decreased levels.

Odds ratios, adjusted for age, sex, and BMI, were calculated for a one-SD change in metabolite concentration for each prediabetic state versus normal. The most significant and cross-cohort–reproducible ORs are shown in Table 3 (a complete listing is found in Supplementary Tables 2 and 3). By *P* value rank, the top metabolites associated with iIGT in RISC are α-HB, L-GPC, X-12063, oleic acid, β-HB, and glycine. None of these have a significant association with iIFG except L-GPC (*P* = 0.03). In the case of iIFG, the top metabolites are α-KB, 3-MOB, 2-AAA, 4-MOP, and 3-HIB. Combined IFG and IGT–associated metabolites are α-KB, α-HB, 3-HIB, 2-AAA, 3-MOB, and L-GPC. The top metabolites associated with iIGT in DMVhi are α-HB, α-KB, α-KG, oleic acid, trigonelline, and L-GPC. L-GPC and trigonelline do not show a significant association with iIFG, whereas α-KB and α-KG do, and α-HB is marginally significant (*P* = 0.05). The top metabolites for iIFG are 4-MOP, X-12063, α-KG, 3-MOB, and α-KB. The top metabolites for combined IFG and IGT are α-KB, α-KG, α-HB, 3-HIB, oleic acid, and L-GPC. The metabolites that were significantly associated with iIGT in both cohorts are α-HB, α-KB, oleic acid,

Table 1—RISC: anthropometric and metabolic characteristics by glycemic status

	Normal	iIGT	iIFG	IFG and IGT
n	623	56	220	56
Women (%)	60	57	38§,‖	55
Age (years)	46 ± 8	50 ± 8*	50 ± 9†	51 ± 9‡
Familial diabetes (%)	24	32	35§	46§
BMI (kg/m^2)	25.0 ± 4	26.0 ± 4*	27.0 ± 4†	28.0 ± 5†
Systolic blood pressure (mmHg)	119 ± 15	123 ± 11*	124 ± 15†	122 ± 13
HDL cholesterol (mg/dL)	58.2 ± 16	52.4 ± 14*	54.9 ± 15*	51.9 ± 16*
Triglycerides (mg/dL)	77 (47)	83 (79)*	93 (57)†	99 (63)†
FFA (mmol/L)	0.54 (0.29)	0.71 (0.28)†	0.55 (0.23)¶	0.72 (0.30)†
Fasting insulin (mU/L)	4.7 (3.3)	5.2 (4.5)	6.3 (4.2)†	6.7 (6.2)†
2-h glucose (mg/dL)	96 ± 21	156 ± 14†	108 ± 20†,¶	159 ± 15†
Fasting glucose (mg/dL)	88 ± 9	92 ± 6‡	106 ± 5†,¶	108 ± 5†
Glucose sensitivity (pmol · min^{-1} · m^{-2} · mM^{-1})	146 ± 99	81 ± 28†	121 ± 59*,¶	85 ± 41†
Oral glucose insulin sensitivity (mmol · min^{-1} · kg$_{FFM}^{-1}$)	12.2 ± 2.6	8.87 ± 1.8†	9.87 ± 1.8†,#	8.06 ± 1.6†
Quantose IR score M^Q (29)	7.26 ± 2.0	5.86 ± 1.8†	6.25 ± 1.6†	4.92 ± 1.2†
HOMA-IR (mU/L · mg/dL)	1.2 ± 0.7	1.5 ± 1.0†	1.9 ± 1.0‡,¶	2.5 ± 1.8†

Data are mean ± SD or median (interquartile range). *P* values calculated in JMP 9.0. Normal = NFG and NGT. IR, insulin resistance. *$P < 0.05$ vs. normal. †$P < 0.0001$ by Wilcoxon rank sum test. ‡$P < 0.001$. §$P < 0.05$ by χ^2 test. #iIFG vs. iIGT: $P < 0.05$. ¶$P < 0.0001$ by Wilcoxon rank sum test. ‖$P < 0.05$ by χ^2 test.

L-GPC, and serine. The metabolites most strongly associated with iIGT versus iIFG in both cohorts are α-HB, oleic acid, L-GPC, and serine (which is not associated with combined IFG and IGT). The metabolites most strongly associated with iIFG versus iIGT in both cohorts are 3-MOB and 4-MOP. α-KB is significantly associated with iIGT, iIFG, and combined IFG and IGT in both cohorts.

α-HB, L-GPC, and oleic acid underwent additional evaluation as potential biomarkers of IGT in the Botnia cohort. Quantitative measurements of these three metabolites were made, and ORs for them, as calculated before, are shown in Table 3. α-HB and oleic acid are strongly associated with both iIGT and combined IFG and IGT (*P* values <1E-9) and have no significant association with iIFG. L-GPC is strongly associated with iIGT (*P* = 5.8E-11) and combined IFG and IGT (*P* = 0.002) but is also associated with iIFG (*P* = 0.002). L-GPC, however, has ORs <1 in iIGT and combined IFG and IGT and an OR >1 in iIFG.

Likelihood ratio tests indicated that there were significant differences in the effect size of the associations. The OR for α-HB in DMVhi for iIGT (2.75) was significantly different ($P < 0.033$) from iIFG (1.31); for L-GPC in RISC, the OR for iIGT (0.48) was significantly different ($P < 0.045$) from iIFG (0.82). The OR for L-GPC in Botnia for iIGT (0.48) versus iIFG (1.17) was highly significant, as expected from the reverse direction of the association ($P < 0.0001$). For 3-MOB in RISC, the OR for iIGT (1.49) versus iIFG (1.45) was marginally significant ($P < 0.05$). Finally, for oleic acid in DMVhi, the OR for iIGT (1.83) versus iIFG (1.36) was significantly different ($P < 0.03$).

IGT Prediction Models

The AUCs for multivariate models for the prediction of IGT as a categorical variable in the subjects without diabetes in RISC, DMVhi, and Botnia are shown in Table 4. In each cohort, the model based on age, sex, BMI, and FPG is compared with the model containing the same variables plus α-HB, L-GPC, and oleic acid. There are significant increases in AUC upon addition of the three metabolites to the age, sex, BMI, and FPG model in each cohort (RISC, +0.10; DMVhi, +0.05; Botnia, +0.06). Each metabolite makes a significant contribution to the model in each cohort except for oleic acid in DMVhi. The models were also evaluated for their ability to predict combination IFG and IGT within the subset of subjects with IFG in each cohort (Table 4). Again the three metabolites added significantly to the AUCs (RISC, +0.09; DMVhi, +0.08; Botnia, +0.05), although the AUCs were, overall, lower in the subjects with IFG due to poorer performance of the age/sex/BMI/FPG component (especially in the DMVhi cohort). Finally, addition of family history to the RISC study models of age, sex, BMI, and FPG or to the model containing the same variables plus α-HB, L-GPC, and oleic acid did not change the AUCs.

CONCLUSIONS

This study identified α-HB, L-GPC, and oleic acid as selective and reproducible metabolite biomarkers of iIGT, whereas 3-MOB and 4-MOP were found to be selective and reproducible metabolite biomarkers of iIFG. Subjects with combined IFG and IGT were found to have metabolite profiles reflecting aspects of both iIFG and iIGT. α-KB was identified as a general marker of IFG and IGT.

In general, iIFG and iIGT are both characterized by a stronger family history of diabetes; higher BMI, systolic blood pressure, and triglycerides; and lower HDL levels with respect to NGT, but these anthropometric and metabolic characteristics do not clearly distinguish between the prediabetic states. Insulin resistance, as measured by the clamp technique, is worse in iIGT than iIFG (M_{wb} = normal [*n* = 922] 7.54 mg · min^{-1} · kg^{-1}, iIFG [*n* = 173] 6.74, and iIGT [*n* = 77] 4.78 [$P < 0.001$ vs. normal and iIFG], unpublished baseline data from RISC) as is also oral glucose insulin sensitivity (an OGTT-based estimate of M_{wb}) (Table 1). In addition, in RISC, β-cell glucose sensitivity is also significantly lower in iIGT (and combined IFG and IGT) than in iIFG or normal subjects. In contrast, HOMA of insulin

Table 2—Metabolite levels by glycemic status: RISC

Metabolite	Class	Normal	iIGT	iIFG	IFG and IGT
α-HB	Methionine/threonine metabolism	3.58 (1.6)	4.90 (2.4)†	3.88 (1.6)‡,§	4.72 (1.7)†,¶
α-KB	Methionine/threonine metabolism	0.343 (0.24)	0.442 (0.42)‡	0.448 (0.21)†	0.530 (0.27)†,#,*
α-KG	Krebs cycle intermediate	0.964 (0.32)	0.994 (0.50)	0.954 (0.27)	1.07 (0.32)#
β-HB	Ketone body	4.13 (5.1)	5.59 (9.5)‡	3.86 (3.9)‖	5.31 (5.7)‡,#
2-AAA	Lysine metabolism	0.0754 (0.037)	0.0910 (0.040)‡	0.0894 (0.036)†	0.106 (0.047)†,#
3-HIB	Valine metabolism	1.21 (0.47)	1.40 (0.55)‡	1.32 (0.45)‡	1.47 (0.62)†,#
3-MOB	Valine metabolism	1.59 (0.46)	1.70 (0.72)‡	1.77 (0.39)†	1.83 (0.46)†,*
3-MOP	Isoleucine metabolism	2.43 (0.94)	2.82 (1.4)‡	2.66 (1.2)‡	2.63 (0.87)‡
4-MOP	Leucine metabolism	3.75 (1.3)	4.07 (1.8)	4.26 (1.2)†	3.99 (1.5)‡
Creatine	Creatine metabolism	3.60 (2.9)	4.20 (4.8)‡	3.78 (2.6)‖	4.98 (3.4)#
Glycine	Amino acid	18.2 (6.8)	15.8 (5.3)‡	17.3 (6.1)‖	16.1 (4.7)‡
Hydroxyisovaleroyl carnitine	Leucine metabolism	0.0254 (0.012)	0.0279 (0.013)	0.0277 (0.011)‡	0.0271 (0.010)
Isoleucine	BCAA	6.74 (1.9)	6.95 (2.0)	7.38 (2.1)†	7.22 (1.9)‡
Leucine	BCAA	13.5 (3.6)	13.8 (3.4)	14.6 (3.5)‡	14.8 (3.6)‡
L-GPC	Lyso-phosphocholine	15.9 (7.3)	11.9 (6.2)†	14.9 (6.6)‡,‖	11.8 (5.0)†,¶
Oleic acid	Fatty acid	48.5 (27)	60.8 (31)†	48.8 (25)§	57.7 (28)†,¶
Phenylalanine	Aromatic amino acid	8.49 (1.3)	8.43 (1.4)	8.83 (1.4)‡,‖	9.15 (1.9)‡,*
Serine	Amino acid	10.5 (2.6)	9.37 (3.2)‡	10.2 (2.4)‖	10.3 (2.9)
Trigonelline	Nicotinate metabolism	0.0899 (0.16)	0.0676 (0.095)	0.101 (0.14)‖	0.094 (0.16)
Tyrosine	Aromatic amino acid	9.73 (2.5)	9.51 (2.9)	10.4 (2.3)†,‖	10.8 (2.7)†,*
Valine	BCAA	23.3 (5.9)	23.9 (5.6)	25.0 (5.1)†	26.2 (5.2)‡,*
Vitamin B5 (pantothenic acid)	Vitamin; precursor to CoA	0.0330 (0.013)	0.0380 (0.018)‡	0.0338 (0.014)	0.0370 (0.017)‡,#
X-12063	Unknown	0.197 (0.20)	0.319 (0.32)†	0.266 (0.22)†	0.331 (0.34)†,#

All values in mg/L except X-12063, which equals area ratio. Data are median (interquartile range). ‡$P < 0.05$, vs. normal. †$P < 0.0001$. ‖iIFG vs. iIGT: $P < 0.05$. §$P < 0.0001$. #iIFG vs. IFG and IGT: $P < 0.05$. ¶$P < 0.0001$. *iIGT vs. IFG and IGT: $P < 0.05$ by the Wilcoxon rank sum test.

resistance (HOMA-IR) is significantly higher in iIFG than iIGT in RISC and DMVhi (but not in Botnia). This finding has been reported previously in the Metabolic Syndrome in Men (METSIM) study (26). Overall, these data confirm greater peripheral insulin resistance in IGT and greater hepatic insulin resistance in IFG (27). In terms of T2DM predictivity, in a prospective, population-based study, iIFG (by the American Diabetes Association definition) had a similar relative risk of progression (~3.0) as iIGT, but a 2.5-fold lower population-attributable risk on account of its lower prevalence in the population. IGT with any fasting glucose, on the other hand, had a sixfold increased relative risk of progression (5).

Several metabolites associated with IFG, controlled for age and BMI, were reported previously from nontargeted metabolomics using semiquantitative data in the TwinsUK study (14), in which, however, glucose tolerance was not assessed. These metabolites included several that were quantitated in this study: three branched-chain α-keto acids (BCKAs) (3-MOB, 3-MOP, and 4-MOP), two BCAAs (isoleucine and leucine), and α-HB. Specifically, 3-MOP was reported to be the strongest predictor of IFG; this finding was replicated in a second cohort (KORA F4). Of these IFG markers, 3-MOB and 4-MOP have the strongest associations with iIFG in the current study, whereas all are associated with combined IFG and IGT. The α-HB association with IFG in TwinsUK may be driven by subjects with combined IFG and IGT, as is seen here.

A second study (the KORA cohort) using targeted metabolomic profiling with 188 metabolites reported 3 metabolites associated with IGT: acetylcarnitine, L-GPC, and glycine; the latter 2 were replicated in the EPIC cohort (12). Our data replicate the finding with L-GPC and glycine (RISC only), whereas acetylcarnitine was not measured. A third study found α-HB to be a predictive biomarker of the 1-h plasma glucose value from the OGTT in a cohort of subjects at increased risk of diabetes (28). Finally, we recently reported that α-HB is correlated with 2hPG and is a predictive biomarker of IGT in RISC and DMVhi (15).

We previously reported that α-HB, L-GPC, and oleic acid are biomarkers of insulin sensitivity as measured by the hyperinsulinemic-euglycemic clamp in the RISC study at baseline (25,29). In a subsequent report, we showed that α-HB and L-GPC were predictors of incident dysglycemia in RISC and of incident T2DM in Botnia (13). These two metabolites had similar predictive power for dysglycemia as 2hPG when included in multivariate models comprised of age, sex, BMI, FPG, and family history of diabetes. The current findings close the loop by showing that these molecules are strongly associated with IGT, and independently predict its development from NGT (Botnia), precisely because of their relation to insulin resistance.

α-HB is a shunt metabolite with no known metabolism other than its production from and conversion back to α-KB mediated by lactate dehydrogenase (LDH) (30) (Supplementary Fig. 1). α-KB is reported to be a substrate for two key catabolic α-ketoacid dehydrogenase complexes, pyruvate dehydrogenase (PD) and branched-chain α-keto acid dehydrogenase (BCKD), by virtue of its structural analogy with the primary substrates for each complex, pyruvate,

Table 3—Key metabolite data by cohort: ORs of iIGT, iIFG, or IFG and IGT versus normal controlled for age, sex, and BMI

		iIGT		iIFG		IFG and IGT	
Study	Metabolite	OR (95% CI)	Adjusted *P*	OR (95% CI)	Adjusted *P*	OR (95% CI)	Adjusted *P*
RISC	α-HB	2.54 (1.86–3.48)	5.1E-9	0.99 (0.83–1.18)	0.09	2.28 (1.66–3.14)	4.1E-7
	L-GPC	0.48 (0.35–0.66)	4.6E-6	0.82 (0.68–0.98)	0.03	0.46 (0.33–0.64)	3.5E-6
	Oleic acid	1.87 (1.38–2.55)	6.4E-5	1.01 (0.85–1.21)	0.9	2.07 (1.49–2.87)	1.3E-5
	Serine	0.62 (0.46–0.82)	0.0009	1.03 (0.87–1.22)	0.7	0.93 (0.69–1.24)	0.6
	α-KB	1.46 (1.10–1.95)	0.01	1.50 (1.26–1.78)	4.1E-6	2.39 (1.75–3.25)	3.0E-8
	3-MOB	1.49 (1.11–2.01)	0.009	1.45 (1.22–1.74)	4.0E-5	2.15 (1.57–2.94)	1.9E-6
	4-MOP	1.39 (1.00–1.94)	0.06	1.36 (1.11–1.65)	0.003	2.01 (1.41–2.85)	9.7E-5
DMVhi	α-HB	2.75 (1.81–4.19)	3.6E-5	1.31 (1.04–1.66)	0.05	2.36 (1.66–3.35)	3.0E-5
	L-GPC	0.55 (0.36–0.84)	0.03	0.97 (0.76–1.24)	0.86	0.45 (0.32–0.64)	1.0E-4
	Oleic acid	1.83 (1.23–2.71)	0.01	1.36 (1.08–1.70)	0.03	1.91 (1.37–2.66)	1.0E-4
	Serine	0.63 (0.43–0.93)	0.04	1.06 (0.85–1.33)	0.67	0.81 (0.59–1.13)	0.28
	α-KB	1.93 (1.29–2.89)	0.01	1.52 (1.21–1.93)	0.01	2.58 (1.82–3.66)	<1.0E-5
	3-MOB	1.25 (0.81–1.91)	0.4	1.38 (1.07–1.77)	0.03	1.69 (1.17–2.43)	0.02
	4-MOP	1.11 (0.71–1.70)	0.71	1.65 (1.26–2.17)	0.001	1.90 (1.30–2.77)	0.004
Botnia	α-HB	2.03 (1.65–2.49)	2.5E-11	1.01 (0.92–1.11)	0.8	1.78 (1.53–2.08)	6.8E-13
	L-GPC	0.49 (0.40–0.61)	5.8E-11	1.17 (1.06–1.29)	0.002	0.77 (0.66–0.90)	0.002
	Oleic acid	1.90 (1.55–2.33)	9.6E-10	1.03 (0.94–1.13)	0.6	1.69 (1.45–1.98)	5.7E-11

and the trio of 3-MOB, 3-MOP, and 4-MOP, respectively (31). PD is important in metabolic flexibility and is a key control point for determining the use of glucose or fatty acids for oxidative phosphorylation in a given tissue (32). PD activity is decreased in T2DM (33) and may be decreased in IGT as well. The increases in α-HB and α-KB seen in IGT could be a reflection of decreased PD activity and decreased flux of α-KB through it. In support of this concept are the increased levels of free fatty acids (FFAs) seen in this study in both IGT and iIGT, as they are known to decrease PD activity through their oxidation to acetyl-CoA, a known regulator of PD (33,34). This possibility is supported by the raised levels seen here in IGT of the ketone body and biomarker of fatty acid oxidation, β-HB. In RISC, α-HB levels are correlated with both FFAs (r = 0.31, $P < 0.0001$) and β-HB (r = 0.44, $P < 0.0001$). In addition, α-HB levels are known to be elevated with extended fasting (35), another state characterized by decreased PD activity (33). α-KB levels could also be a function of the rate of methionine and/or threonine catabolism. Indeed, oxidative stress could increase methionine catabolism via cystathionine in order to produce cysteine for glutathione synthesis while also producing α-KB (36).

BCKD carries out a key step in BCAA catabolism. The increased levels of BCKD substrates (particularly 3-MOB, 4-MOP, and α-KB) seen in iIFG may reflect decreased BCKD activity in this glycemic state as has been reported in T2DM (37). The finding that α-HB tracks with α-KB in iIGT but not in iIFG suggests some subtle differences in their interconversion ratio in these two glycemic states. It would only take a small change to increase α-KB without increasing α-HB, as the ratio favors α-HB (typically ~10:1).

PD and BCKD are closely related enzyme complexes (38) present in a number of metabolically active tissues (e.g., muscle, liver, and adipose); they are regulated by a number of common factors, including insulin. It is possible that they both play a role in affecting plasma α-HB and α-KB levels, although their relative activities and tissue specificities may well differ in iIFG and iIGT.

The BCAAs have an extensive literature of being associated with obesity, insulin resistance, T2DM, and incident T2DM (39). In this study, however, BCAAs were only modestly associated with iIFG (DMVhi only) and with combined IFG and IGT and were not associated with iIGT. Of the 23 metabolites in our profiling panel, the BCAAs are among the highest correlated with HOMA-IR, insulin levels, and BMI; similar correlations have been noted in the Framingham Offspring Study (10). In the multivariate predictive models of RISC and DMVhi data, insulin and BMI as covariates very likely confound the link between BCAAs and glycemic category.

The strength of this study relies on the 4,053 subjects from three cohorts evaluated and the reproducible findings that α-HB, L-GPC, and oleic acid are associated with IGT. A limitation of the study is its lack of diversity, as all three cohorts evaluated are European Caucasians, and further studies in diverse populations are necessary to understand the breadth of the metabolite finding to dysglycemic states. In addition, all three cohorts are subsets of larger cohorts and it is possible that some selective bias has

Table 4—AUCs for prediction of IGT and IFG and IGT in RISC, DMVhi, and Botnia

	RISC	DMVhi	Botnia
Model (all cohort subjects without diabetes)			
n (% IGT)	955 (11.7%)	668 (11.8%)	2,430 (15.9%)
Age/sex/BMI/FPG	0.716	0.814	0.722
Age/sex/BMI/FPG/α-HB/L-GPC/oleic acid	0.813*	0.863†	0.786‡
Model (IFG subject subcohorts only)			
n (% IFG and IGT)	276 (20.3%)	152 (31.6%)	1,197 (21.6%)
Age/sex/BMI/FPG	0.692	0.681	0.718
Age/sex/BMI/FPG/α-HB/L-GPC/oleic acid	0.780§	0.760‖	0.770¶

*P value vs. age/sex/BMI/FPG: 1.3E-5. †9.9E-4. ‡1.1E-10. §1E-2. ‖3E-2. ¶1E-6.

been introduced. On the other hand, the reproducible findings across three cohorts may serve to mitigate this possibility.

In the RISC study, which is the most representative of European Caucasians of the three cohorts reported here, cut points can be calculated for α-HB, L-GPC, and oleic acid to achieve 80% sensitivity for the identification of IGT subjects. These cut points (and % specificity) are as follows: α-HB (≥3.84 mg/L, 55%), L-GPC (<16 mg/L, 48%), and oleic acid (>47 mg/L, 47%). We recently reported a novel test for IGT (15) based on a multimetabolite model that includes α-HB, L-GPC, oleic acid, and FPG that was developed in subjects from the RISC 3-year follow-up and validated in subjects from the DMVhi 3-year follow-up. In DMVhi, using the top tertile cutoff of the test scores, the sensitivity and specificity of the test were 78 and 73%, respectively, for the identification of IGT subjects.

In summary, the current results define circulating metabolite patterns that distinguish iIFG from iIGT above and beyond anthropometric and metabolic differences. They also validate the 23-metabolite panel as a tool to profile and stratify prediabetic states. These findings can be used to generate new predictive models for the identification of subjects with, or at risk for, IGT without performing an OGTT.

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