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Metformin Effect on Nontargeted Metabolite Profiles in Patients With Type 2 Diabetes and in Multiple Murine Tissues

Diabetes 2016;65:3776–3785 | DOI: 10.2337/db16-0512

Metformin is the first-line oral medication to increase insulin sensitivity in patients with type 2 diabetes (T2D). Our aim was to investigate the pleiotropic effect of metformin using a nontargeted metabolomics approach. We analyzed 353 metabolites in fasting serum samples of the population-based human KORA (Cooperative Health Research in the Region of Augsburg) follow-up survey 4 cohort. To compare T2D patients treated with metformin (mt-T2D, *n* = 74) and those without antidiabetes medication (ndt-T2D, *n* = 115), we used multivariable linear regression models in a cross-sectional study. We applied a generalized estimating equation to confirm the initial findings in longitudinal samples of 683 KORA participants. In a translational approach, we used murine plasma, liver, skeletal muscle, and epididymal adipose tissue samples from metformin-treated *db/db* mice to further corroborate our findings from the human study. We identified two metabolites significantly (*P* < 1.42E-04) associated with metformin treatment. Citrulline showed lower relative concentrations and an unknown metabolite X-21365 showed higher relative concentrations in human serum when comparing mt-T2D with ndt-T2D. Citrulline was confirmed to be significantly (*P* < 2.96E-04) decreased at 7-year follow-up in patients who started metformin treatment. In mice, we validated significantly (*P* < 4.52E-07) lower citrulline values in plasma, skeletal muscle, and adipose tissue of metformin-treated animals but not in their liver. The lowered values of citrulline we observed by using a nontargeted approach most likely resulted from the pleiotropic effect of metformin on the interlocked urea and nitric oxide cycle. The translational data derived from multiple murine tissues corroborated and complemented the findings from the human cohort.

Metformin became the first-line choice for treatment of type 2 diabetes (T2D) in the course of the UK Prospective Diabetes Study (UKPDS) (1). Additionally, metformin has

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been reported to have other pleiotropic effects; e.g., it reduces insulin resistance (2), improves the uptake of glucose in muscle (3,4), reduces the risk for cancer (5), and lowers the values of LDL cholesterol (LDL-C) (6). The underlying mechanism of the reduction of LDL-C is, at least in part, due to the activation of the AMPK in the liver (7). Apart from that, AMPK affects several processes such as nitric oxide (NO) production by endothelial NO synthase (eNOS) (8), which is also stimulated by metformin (9). However, the mode of action of metformin is not completely understood (10–12).

Our previous study was based on a targeted metabolomics approach to explore the effects of metformin on lipid profiles in the population-based KORA (Cooperative Health Research in the Region of Augsburg) cohort (6,13,14). Irving et al. (15) recently reported decreased levels of arginine and citrulline as an effect of insulin sensitizer therapy in 12 metformin- and pioglitazone-treated individuals and 13 placebo-treated control subjects. Nontargeted metabolomic measurements have been applied to investigate hyperglycemia (16,17) and the effects of metformin treatment in individuals without diabetes (18). However, none of the previous nontargeted metabolomics studies investigated metformin treatment in patients with T2D.

In this study we focused on serum metabolites associated with metformin treatment based on a nontargeted approach in a human population from the KORA cohort. A cross-link from human to mice was corroborated in multiple tissues (plasma, liver, skeletal muscle, and epididymal adipose tissue) from a mouse study. Biologically relevant pathways for the identified metabolites were analyzed using bioinformatical approaches.

RESEARCH DESIGN AND METHODS

Ethics Statement

All participants gave written informed consent. The KORA study was approved by the ethics committee of the Bavarian Medical Association, Munich, Germany.

Approval for Mouse Study

Within this study, all mice were bred and housed in a temperature- and humidity-controlled environment in compliance with Federation of European Laboratory Animal Science Associations protocols. Animal experiments were approved by the District Government of Upper Bavaria (Regierung von Oberbayern, Gz.55.2–1-54–2531–70–07, 55.2–1-2532–153–11).

KORA Cohort

KORA is a population-based cohort study conducted in southern Germany (14). The baseline survey 4 (KORA S4) consists of 4,261 individuals (aged 25–74 years) examined between 1999 and 2001. During the years of 2006 to 2008, 3,080 individuals took part in the follow-up survey 4 (KORA F4). Clinical data for each participant were retrieved from medical records. On the basis of fasting glucose, 2-h postglucose load, and physician-validated and self-reported diagnoses, KORA participants were classified according to the World Health Organization diagnostic criteria. A further grouping of patients with T2D was based on information on medication (19,20) (Table 1). Only participants with metabolite measurements were included in the present analysis (Metabolon, n = 1,768 in KORA F4). We excluded *1*) participants with overnight nonfasting serum samples (n = 8), *2*) patients suffering from type 1 diabetes and drug-induced (e.g., via steroids) diabetes (n = 6), *3*) T2D patients treated with insulin (n = 16) or both insulin and metformin (n = 13), and *4*) patients taking glucose-lowering oral medication other than metformin (n = 17). Furthermore, participants with isolated impaired fasting glucose (IFG) (n = 77) were excluded. We have previously shown that IFG and impaired glucose tolerance (IGT) should be considered as two different phenotypes (21).

In KORA F4, we focused on four groups: *1*) participants with normal glucose tolerance (NGT), *2*) individuals with prediabetes with IGT, *3*) T2D patients without glucose-lowering treatment (non-antidiabetes drug treated, ndt-T2D), and *4*) metformin-treated T2D (mt-T2D) patients (Table 1).

The same exclusion and classification criteria were used in the longitudinal analyses. We only considered participants with metabolite measurements in both studies (KORA S4 and F4, n = 818), and we excluded at both time points *1*) participants with overnight nonfasting serum samples (n = 88), which included patients suffering from type 1 diabetes or drug-induced diabetes, *2*) participants taking oral glucose-lowering medication other than metformin (n = 11), *3*) participants undergoing insulin treatment (n = 3), and *4*) participants with a missing diabetes status (n = 33). The remaining 683 participants were ndt-T2D individuals with prediabetes and healthy control subjects at KORA S4, 37 of whom started metformin treatment at KORA F4.

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Received 21 April 2016 and accepted 1 August 2016.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db16-0512/-/DC1.

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See accompanying article, p. 3537.

Table 1—Characteristics of the KORA F4 cross-sectional study samples (n = 1,604)

Clinical parameters	NGT	IGT	ndt-T2D	mt-T2D
n	1,143	272	115	74
Age, years	58.9 (8.5)	63.8 (8.1)	65.1 (7.1)	66.2 (7.5)
Male, %	45	50	61	58
BMI, kg/m^2	27.0 (4.3)	29.8 (4.7)	31.0 (4.8)	32.0 (5.6)
Waist, cm	92 (12.9)	100.2 (14.3)	104.9 (12.1)	106.7 (13.0)
Physical activity, % >1 h per week	63	54	48	36
High alcohol intake, %*	20	18	25	19
Smoker, %	18	8	12	15
Systolic BP, mmHg	121.6 (17.7)	129.0 (19.0)	134.3 (19.2)	130.7 (18.2)
HDL-C, mg/dL	58.9 (14.8)	54.4 (14.2)	49.6 (11.5)	50.4 (9.6)
LDL-C, mg/dL	140.7 (34.3)	144.5 (36.2)	136.6 (36.0)	124.1 (28.3)
Total cholesterol, mg/dL	223.4 (37.7)	226.4 (41.5)	214.1 (37.0)	203.8 (37.8)
Triglycerides, mg/dL	118.5 (84.6)	150.7 (89.0)	172.5 (128.7)	177.5 (140.5)
HbA_{1c}, %	5.4 (0.3)	5.6 (0.3)	6.3 (0.9)	6.8 (1.1)
HbA_{1c}, mmol/mol	35.7 (3.2)	38.1 (3.9)	44.91 (9.9)	51.2 (11.6)
Fasting glucose, mg/dL	93.2 (7.5)	101.2 (10.6)	126.6 (30.5)	142.0 (36.0)
2-h postglucose load, mg/dL	100.8 (20.6)	162.6 (17.5)	216.0 (50.7)†	
Time since diagnosis, years			1.4 (2.6)‡	7.4 (6.6)
Insulin, μIU/mL	7.1 (26.1)	10.1 (10.9)	14.3 (14.3)	11.6 (11.0)
Leptin, ng/mL	17.2 (19.2)	24.2 (21.3)	26.8 (21.2)	27.8 (25.0)
Statin usage, %	12	15	28	36
β-Blocker usage, %	16	31	43	38
ACE inhibitor usage, %	10	21	32	45
ARB usage, %	8	10	17	14
Insulin therapy, %	0	0	0	0
Metformin usage, %	0	0	0	100
Parental T2D, %	28	30	37	49

KORA F4 study characteristics (including solely subjects with available Metabolon measurements). Percentages of individuals or means (SD) are shown for each variable and each group (NGT, IGT, ndt-T2D, and mt-T2D). *≥20 g/day for women; ≥40 g/day for men. †n = 81. ‡For newly diagnosed T2D patients (n = 74), years since T2D diagnosis was defined as 0.

The data from KORA S4 and F4, including metabolite concentrations with clinical phenotypes, are available upon request through the platform KORA-PASST (project application self-service tool) (www.helmholtz-muenchen.de/kora-gen).

Blood Sampling

In the KORA cohort study, blood was drawn into Monovette serum tubes (Sarstedt AG & Co., Nümbrecht, Germany) in the morning between 8:00 A.M. and 10:30 A.M. after at least 8 h of fasting. Tubes were gently inverted twice, followed by resting 30 min at room temperature to obtain complete coagulation. For serum collection, blood was centrifuged at 2,750*g* at 15°C for 10 min. Serum was filled into synthetic straws, which were stored in liquid nitrogen (−196°C) until metabolomic analyses.

Nontargeted Metabolite Profiling

The serum samples from participants of KORA S4 and F4 were measured with the Metabolon analytical system (Metabolon, Inc., Durham, NC). Metabolon applied a nontargeted semiquantitative liquid chromatography–tandem mass spectrometry (LC-MS/MS) and gas chromatography–mass spectrometry (GC-MS) platform for the identification of structurally named and unknown molecules (22,23). We measured 363 (including 109 unknown) metabolites in fasting serum samples from KORA S4. In the 7-year KORA F4, 353 metabolites (including 107 unknown) were determined (24).

In this study, we applied the same criteria for quality control as described by Albrecht et al. (25). In brief, metabolites with more than 20% missing values were excluded, as were samples with more than 10% missing metabolites (25). All normalized relative ion counts were log transformed, and the remaining data were imputed with Multivariate Imputation by Chained Equations (MICE) (26). We used 363 metabolites in KORA S4 and 353 metabolites in KORA F4 (Supplementary Table 1). The number of overlapping metabolites in KORA S4 and

F4 was 312. Metabolite names were used according to Shin et al. (27); however, the identity of metabolite ID M32654 and the molecule "3-dehydrocarnitine*" could not be confirmed. We therefore used the name X-21365 (Supplementary Table 1).

Each metabolite was standardized with a mean of zero and an SD of one in each study after the exclusion of nonfasting participants.

Metformin Mouse Intervention Study

Pharmacological studies were conducted in 20 male 8-week-old diabetic BKS.Cg-*Dock7*$^{m+/+}$ *Lepr*^{db}/J (*db/db*) mice that were bred and housed in a temperature- and humidity-controlled environment in compliance with Federation of European Laboratory Animal Science Associations protocols. To exclude estrous cycle–related influences on metabolic parameters, only male mice were included in this study. From age 3 weeks, all mice were fed a high-fat diet (S0372-E010; ssniff Spezialdiäten, Soest, Germany) containing (gm%) palm fat (13.5), sunflower oil (13.5), starch (30), saccharose (10), casein (20), lignocellulose (5), mineral+vitamin mix (5+2), safflower oil (0.5), and linseed oil (0.5) to manifest a uniform diabetic phenotype. Animals received either vehicle (5% solutol/95% hydroxyethylcellulose) without (n = 10) or with metformin (300 mg/kg; Sigma Aldrich, Taufkirchen, Germany; n = 10) via gavage once daily between 5:00 and 6:00 P.M. before dark-phase onset (6:00 P.M.) for 14 days. At 18 ± 2 h after the last treatment, 4-h fasted mice were sacrificed with an isoflurane overdose, and organs and blood were immediately collected (4). Murine plasma was prepared from whole blood by centrifugation at 4°C, and tissues were freeze-clamped; both were stored at −80°C until further analyses. All samples were measured with the Metabolon analytical system. Metabolites with more than 20% missing values were excluded, as were samples with more than 10% missing metabolites (25). All normalized relative ion counts were log transformed, and the remaining data were imputed with MICE (26). Linear regression was done on metabolite values for metformin-treated mice as the cases as well as for the nonmetformin-treated, vehicle-gavaged mice as the controls. A metabolomics examination was done for plasma, liver, skeletal muscle, and epididymal adipose tissue (Table 5 and Fig. 1*B*).

Statistical Analysis

To evaluate the effect of metformin treatment on certain metabolites, multivariable linear regression models were conducted with the relative metabolite concentration values as outcome and the grouping variable as predictor. Each metabolite was assessed individually. To consider potential risk factors and confounding parameters with known effect on metabolite profiles (6,13,28–32), two models were used: *1*) adjusted for age and sex as the crude model and *2*) adjusted for age, sex, BMI, physical activity, high alcohol intake, smoking status, systolic blood pressure (BP), HbA_{1c}, fasting glucose, HDL cholesterol (HDL-C), and triglycerides as well as the use of statins, β-blockers, ACE inhibitors, and angiotensin receptor blockers (ARB) as the full model. The association of conventional risk factors of T2D as well as other population characteristics with metformin treatment was calculated via χ^2 test for categorical variables. Shapiro–Wilk test was applied to test continuous variables for normal distribution ($P \leq 0.05$ for nonnormally distributed variables, $P > 0.05$ for normally distributed variables), followed by Student's t test for normally distributed continuous variables and Wilcoxon test for nonnormally distributed continuous variables.

To account for multiple testing for the linear models, Bonferroni correction was applied, and only metabolites with $P < 0.05/353 = 1.42E\text{-}04$ were considered to be statistically significantly different in KORA F4. In addition, we calculated the adjusted P value with the false discovery rate (FDR) using the Benjamini-Hochberg method, which is not as stringent as the Bonferroni correction. For the full linear models, participants were excluded because of missing information of considered confounders. This led to 1,138 NGT (after exclusion of five individuals because of missing confounding information), 272 IGT, 114 ndt-T2D (after exclusion of one individual because of missing confounding information), and 70 mt-T2D (after exclusion of four individuals because of missing confounding information) participants.

In the KORA S4 to F4 longitudinal study (S4 → F4), generalized estimating equations (GEE) were used to validate the significant metabolites in both crude and full models.

All statistical analyses were performed in R (version 3.2.2) (33).

Pathway Analysis

Pathways were explored using databases, considering tissue and organ specificity. The link from observed significant metabolites to the interacting enzymes was drawn using the Human Metabolome Database (34). Protein–protein interactions were analyzed with the Search Tool for the Retrieval of Interacting Genes (35) and the Kyoto Encyclopedia of Genes and Genomes (36). To consider drug-related effects of metformin on certain targets, we used DrugBank (37). The link between metformin targets and the protein network was analyzed using the Kyoto Encyclopedia of Genes and Genomes (36).

RESULTS

Population Characteristics of Human and Mouse Studies

On the basis of the available nontargeted metabolomic profiles, our human discovery study, KORA F4, includes 1,143 NGT, 272 IGT, 115 ndt-T2D, and 74 mt-T2D participants (Table 1). Among the four groups, mt-T2D patients were the oldest, were more frequently men, and had the highest values of HbA_{1c}, fasting glucose, triglycerides, BMI, and waist circumference (Table 1).

The longitudinal KORA study includes samples of 683 participants without metformin treatment at baseline,

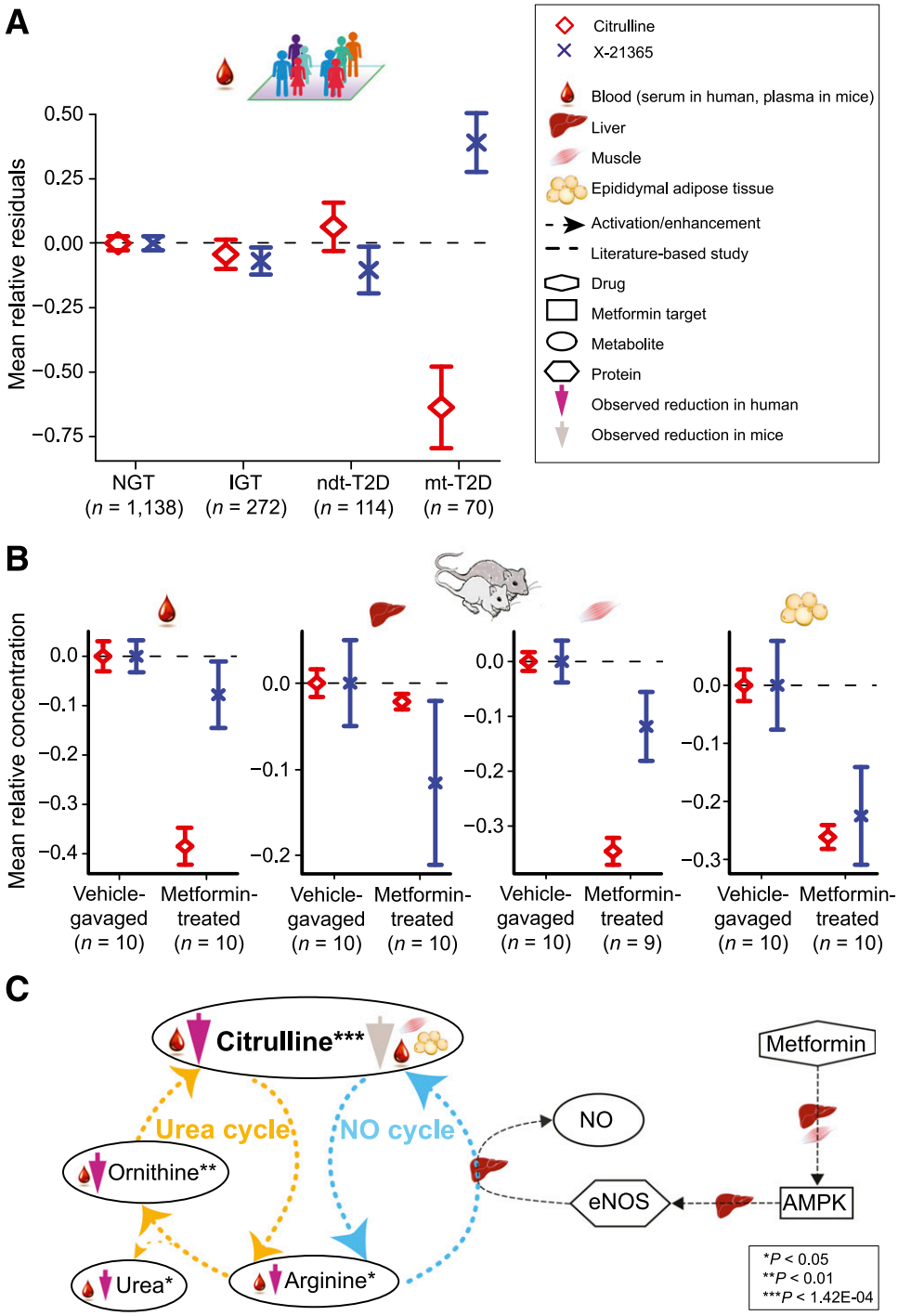

Figure 1—Differences in relative metabolite concentrations in a human study, in a mouse study, and in organ-specific pathways. *A*: Mean relative residuals of the concentrations (with SEM) of two metabolites for the NGT, IGT, ndt-T2D, and mt-T2D groups derived in cross-sectional analysis of KORA F4. Residuals were calculated from a linear regression model with full adjustments. *B*: Mean relative concentrations (with SEM) of two metabolites in four different mouse tissues (plasma, liver, skeletal muscle, and epididymal adipose tissue). *C*: The connections indicated by liver, muscle, and blood (plasma and serum) show organ specificity between metabolites, pathway-related proteins, metformin targets, and metformin. The metabolites (ellipses) were connected to metformin treatment (straight-sided hexagons), proteins (hexagons), and metformin targets (rectangles). The activation/stimulation is indicated with arrows. For further information, see Tables 3–5 and Supplementary Tables 2 and 3.

37 of whom were treated with metformin in the 7-year follow-up (Table 2).

From the metformin-treated mice, we obtained 10 samples for plasma, liver, and epididymal adipose tissue and 9 samples for skeletal muscle. In the same amount of vehicle-gavaged control mice, we obtained 10 samples for plasma, liver, epididymal adipose tissue, and skeletal muscle.

Table 2—Characteristics of the KORA S4 → F4 prospective study samples (*n* = 683)

	KORA S4 w/o metformin§ → KORA F4 w/o metformin§			KORA S4 w/o metformin§ → KORA F4 w/ metformin		
Clinical parameters	S4	F4	*P* value	S4	F4	*P* value
n	646	646		37	37	
Age, years	61.4 (4.2)	68.5 (4.2)		63.4 (3.8)	70.4 (3.9)	
Male, %	51	51		54	54	
BMI, kg/m^2	27.9 (3.9)	28.2 (4.2)	0.2	32.8 (4.3)	32 (4.5)	0.49‖
Waist, cm	93.9 (11.0)	96.8 (11.9)	1.72E-05	106.3 (11.3)	106.7 (12.3)	0.87‖
Physical activity, % >1 h per week	47	57	5.47E-04	32	43	0.47
High alcohol intake, %*	20	19	0.65	27	16	0.4
Smoker, %	13	8	9.00E-03	14	8	0.71
Systolic BP, mmHg	132.1 (18.7)	128.2 (19.6)	3.12E-04	144.9 (18.1)	131.6 (18.3)	2.43E-03‖
HDL-C, mg/dL	59.1 (16.3)	56.7 (14.2)	0.02	53.4 (11.8)	52.4 (7.9)	0.69‖
LDL-C, mg/dL	154.5 (40.8)	142.5 (36.9)	1.86E-08	143.5 (37.6)	123.5 (23.6)	0.02
Total cholesterol, mg/dL	245.5 (42.0)	225.2 (40.7)	2.2E-16	234.1 (41.0)	202.1 (33.6)	5.20E-04
Triglycerides, mg/dL	129.9 (76.4)	132.7 (83.4)	0.56	170.6 (169)	154.5 (159.2)	0.79
HbA_{1c}, %	5.6 (0.3)	5.6 (0.5)	0.53	6.4 (0.9)	6.6 (0.7)	0.14
HbA_{1c}, mmol/mol	37.6 (3.7)	38.0 (5.7)	0.53	46.8 (10.3)	48.3 (7.8)	0.14
Fasting glucose, mg/dL	99.7 (10.9)	100.7 (17.6)	0.53	130.7 (30.1)	129.6 (28.3)	0.9
2-h postglucose load, mg/dL	115.1 (37.1)	126.9 (40.8)	8.34E-09	205.5 (76.8)		
Statin usage, %	10	22	8.05E-10	8	29	0.04
β-Blocker usage, %	17	31	5.16E-09	16	29	0.27
ACE inhibitor usage, %	8	21	2.72E-11	16	51	3.18E-03
ARB usage, %	3	12	3.67E-10	3	14	0.2
Insulin therapy, %	0	0		0	0	
Metformin usage, %	0	0		0	100	
Parental T2D, %	25	25		47	47	

Percentages of individuals or means (SD) of participants (with available Metabolon measurements for KORA S4 and F4) are shown for each variable and each group. w/o, without; w/, with. *>40 g/day in men; >20 g/day in women. §Includes participants with NGT, isolated IFG, IGT, and ntd-T2D. ‖Normally distributed (every other distribution is not normally distributed).

Two Metabolites Are Associated With Metformin Treatment in a Human Cross-sectional Study

Two out of the 353 used metabolites (citrulline and X-21365) were found to be significantly ($P < 1.42E-04$) associated with metformin treatment when comparing mt-T2D with ndt-T2D patients in the cross-sectional KORA F4 study (Table 3 and Fig. 1*A*). Using multivariable linear regression models, we detected negative β-estimates for both the crude (β = –0.75, P = 2.31E-05) and full adjustment (β = –0.79, P = 2.54E-05) for

Table 3—Two human serum metabolites significantly associated with metformin treatment in a cross-sectional analyses (KORA F4)

	Crude linear model mt-T2D (*n* = 74) vs. ndt-T2D (*n* = 115)			Full linear model mt-T2D (*n* = 70)¶ vs. ndt-T2D (*n* = 114)¶		
Metabolite	β (95% CI) per SD	*P* value	FDR	β (95% CI) per SD	*P* value	FDR
Citrulline	−0.75 (−1.09, −0.41)	**2.31E-05**	**2.83E-04**	−0.79 (−1.15, −0.43)	**2.54E-05**	**2.83E-04**
X-21365	0.67 (0.38, 0.96)	**7.54E-06**	**1.42E-04**	0.65 (0.34, 0.97)	**5.20E-05**	**1.42E-04**

Estimates (β) and *P* values for the comparison of 189 participants (74 mt-T2D and 115 ndt-T2D) were calculated using linear regression analysis with the crude and full adjustments. Because of missing confounding information, the models with full adjustment were based on fewer participants. Significant metabolites are highlighted in boldface type with respect to Bonferroni correction ($P < 0.05/353 = 1.42E-04$) or the FDR. ¶After exclusion of individuals because of missing confounding information.

citrulline. Hence, the relative concentration of citrulline is significantly lower in mt-T2D compared with ndt-T2D patients. By contrast, the relative concentration of X-21365 was significantly higher in mt-T2D patients than in ndt-T2D patients (Table 3 and Fig. 1*A*). When applying the FDR, no additional associations were found to be significant in both crude and full models (Supplementary Table 3). When applying a significance cutoff of $P < 0.05$ to the comparison of mt-T2D with ndt-T2D for the models with crude and full adjustment, 44 additional metabolites were found, including ornithine, arginine, and urea (Supplementary Table 3).

Five additional pairwise comparisons between the four groups (NGT, IGT, ndt-T2D, mt-T2D) confirmed that these two metabolites are specific for metformin treatment and not due to the progression of the disease. The relative concentration of citrulline was significantly lower in the mt-T2D than in the NGT and IGT groups, whereas the concentration of X-21365 was significantly higher (Fig. 1*A* and Supplementary Table 2). Consistently, neither of the two metabolites showed a significantly different relative concentration in the pairwise comparisons among the three groups without metformin treatment, i.e., NGT, IGT, and ndt-T2D (Fig. 1*A* and Supplementary Table 2).

The Spearman correlation coefficient between the two metabolites was low ($r = 0.06$). We observed similar associations between the two metabolites with a number of risk factors of T2D ($-0.19 < r < 0.19$) when considering 189 individuals with ndt-T2D and mt-T2D in KORA F4 (Supplementary Fig. 1).

Metformin Treatment Is Associated With Decreased Blood Citrulline Values in a Human Longitudinal Cohort

The two metformin-associated metabolites were further investigated in the prospective KORA study. In 37 patients who started metformin treatment during the 7-year follow-up, citrulline was found to be significantly (Bonferroni cutoff for two identified metabolites $P < 0.05/2 = 0.025$) decreased in longitudinal in both the crude (β = –0.67, P = 2.03E-05) and the full model (β = –0.61, 2.96E-04, Table 4). In the same group, X-21365 was significantly increased in the crude (β = 0.41, P = 5.62E-03) but not in the full model (β = 0.16, P = 0.374, Table 4).

Lower Citrulline Relative Concentrations in Plasma, Skeletal Muscle, and Epididymal Adipose Tissue Confirmed in Metformin-Treated Mice

We observed significantly lower plasma citrulline relative concentrations in *db/db* mice following daily, subchronic metformin treatment compared with the vehicle-gavaged control mice (β = –0.39, P = 2.56E-07, Table 5 and Fig. 1*B*), which is consistent with the results observed in humans. In addition, we found significantly lower values of citrulline in both skeletal muscle (β = –0.35, P = 1.79E-09) and epididymal adipose tissue (β = –0.26, P = 4.52E-07). However, citrulline values in the liver did not differ between the metformin-treated and vehicle-gavaged non–metformin-treated *db/db* animals (β = –0.02, P = 0.258, Table 5 and Fig. 1*B*). Significantly different relative concentrations of X-21365 were not found in plasma, skeletal muscle, epididymal adipose tissue, or liver of metformin-treated mice when compared with the controls (Table 5 and Fig. 1*B*).

DISCUSSION

We found significantly lower values of citrulline and significantly higher values of X-21365 in the serum of T2D patients who underwent metformin treatment compared with the nontreated patients. Additionally, using longitudinal settings, we observed that the values of citrulline significantly decreased in patients after they started metformin treatment during the follow-up. A mouse intervention study using metformin confirmed the lower values of citrulline in plasma, as well as in skeletal muscle and epididymal adipose tissue, but not in liver. Citrulline is a nonproteinogenic amino acid, the product of anabolic and the substrate of catabolic processes (38,39). It is synthesized from arginine by releasing NO, which is involved in the regulation of numerous processes in the nervous system, the immune system, and the cardiovascular system (8). Additionally, citrulline is produced from ornithine in the urea cycle (38). We observed ornithine, urea, and arginine to be lowered in human serum (Fig. 1*C*). Consistently, in our previous study, which was based on a targeted metabolomics approach, ornithine was found to be significantly lower in the metformin-treated T2D patients of the

Table 4—Citrulline remains significantly associated with metformin treatment in human serum in a longitudinal analysis (KORA S4 → F4)

	Crude GEE model mt-T2D (n = 37) vs. nonmetformin-treated (n = 646) participants§			Full GEE model mt-T2D (n = 33)¶ vs. nonmetformin-treated (n = 629)¶ participants§		
Metabolite	β (95% CI) per SD	P value	FDR	β (95% CI) per SD	P value	FDR
Citrulline	−0.67 (−0.98, −0.36)	**2.03E-05**	**1.76E-03**	−0.61 (−0.94, −0.28)	**2.96E-04**	**3.21E-04**
X-21365	0.41 (0.12, 0.69)	**5.62E-03**	0.011	0.14 (−0.17, 0.45)	0.374	0.024

GEE model with crude and full adjustment was used to assess the associations between metformin treatment and metabolite serum values in the longitudinal study of 683 participants with no antidiabetes medical treatment at KORA S4. Of these participants, 37 started metformin treatment after KORA S4. Because of missing confounding information, the models with full adjustment were based on fewer participants. Significant metabolites are highlighted in boldface type with respect to Bonferroni correction ($P < 0.05/2 = 0.025$) and the FDR. §Includes participants with NGT, isolated IFG, IGT, and ntd-T2D. ¶After exclusion of individuals because of missing confounding information.

KORA F4 study. Citrulline was not measured in the targeted panel we used (6).

Metformin activates AMPK in the liver and muscle (7,40). AMPK in turn may stimulate eNOS by its phosphorylation (8,41), which suggests a consequent increase of the NO production in the NO cycle (Fig. 1*C*). It is known that elevated production of NO is reflected by increased values of citrulline in urine (42), as citrulline can be used as a surrogate marker for NO (43). The decreased values of citrulline and its precursors in blood, skeletal muscle, and epididymal adipose tissue, as were observed in our study, are most likely due to an accountable, increased excretion of this metabolite. However, urine samples were not available in this study. To confirm this assumption, further studies are necessary.

Furthermore, the lower values of citrulline and arginine we observed are likely to be a consequence of the activation of eNOS. In the NO cycle, eNOS catalyzes the reaction from arginine to citrulline, thereby releasing NO (9,38). NO in turn has beneficial cardiovascular effects. The reason is that NO influences smooth muscles and activates their relaxation (44). This underlies the clinical practice guidelines, which have recommended the use of metformin as first-line therapy in T2D patients with cardiovascular disease, mainly in patients with observed reduced NO levels (45). Additional intake of citrulline to compensate for the lower values of citrulline and arginine might even increase the beneficial effects of metformin on cardiovascular disease (46).

Additionally, citrulline is synthesized in the urea cycle, which is strongly interlocked with the NO cycle (Fig. 1*C*). In mammals, both cycles primarily take place in the liver, but they also take place in the kidney (47). The same accounts for the NO cycle, in which arginine also plays an important role. In fact, similar effects of metformin on the urea and NO cycle were mentioned by Irving et al. (15). Their study design focused on plasma samples of 25 male overweight or obese participants. Furthermore, all 12 metformin-treated participants were additionally treated with pioglitazone (15). Our findings in multiple tissues of mice that were exclusively treated with metformin and in serum of 189 T2D patients enable a deeper understanding of the underlying mode of action for metformin.

The observation that the citrulline values are not affected in the liver of metformin-treated mice is presumably a consequence of the hepatic localization of the consecutive production of citrulline in both the NO and the urea cycle (38), which conserves a state of equilibrium. This is in line with observations in a recent study (18). Furthermore, significantly decreased ornithine values were found in plasma of individuals without diabetes (18).

Apart from the NO and urea cycles, there are additional physiological processes that produce citrulline. The metabolite is also synthesized from other amino acids. Examples of such precursors are glutamine, which is converted in the enterocytes, proline, and glutamate (38). However, we did not observe any significant concentration difference for these metabolites in our human cohort.

Table 5—Metabolites significantly associated with metformin treatment in mouse models

	In plasma		In liver		In skeletal muscle		In adipose tissue	
Metabolite	β (95% CI) per SD	*P* value	β (95% CI) per SD	*P* value	β (95% CI) per SD	*P* value	β (95% CI) per SD	*P* value
Citrulline	−0.39 (−0.49, −0.28)	**2.56E-07**	−0.02 (−0.06, 0.02)	0.258	−0.35 (−0.41, −0.28)	**1.79E-09**	−0.26 (−0.33, −0.19)	**4.52E-07**
X-21365	−0.08 (−0.24, 0.08)	0.311	−0.12 (−0.34, 0.11)	0.295	−0.12 (−0.27, 0.04)	0.126	−0.23 (−0.46, 0.01)	0.063

Estimates (β) and *P* values for the comparison between metformin-treated ($n = 10$, in skeletal muscle [$n = 9$]) and nontreated mice ($n = 10$) sacrificed at 4 h after the last treatment. Significant metabolites are highlighted in boldface type ($P < 0.05$).

X-21365 was not found to be significantly higher in the fully adjusted longitudinal analyses of the KORA S4 → F4 cohort, although it was significant in both cross-sectional analyses and in the longitudinal analyses with crude adjustment. In mice, we did not observe significant differences of X-21365 in any of the examined tissues. Recent advances in the identification of metabolites spectra suggest that this unknown metabolite (X-21365) might be 5-trimethylaminovalerate and therefore closely related to the gut microbiome, which is in line with a recent study (48). Additional studies using both blood and stool samples have to be conducted to confirm this.

The values of metabolites in humans of the KORA study are influenced by multiple factors such as age, sex, BMI, lifestyle, clinical measurements, and medication (6,13,28–32). We therefore considered these factors in the models underlying our cross-sectional discovery and longitudinal investigations in a human cohort. Considering the mouse study, there was no need for a comparable adjustment, as the animals were kept under strict laboratory conditions.

Because of the physiological similarity, we used data from a mouse study not only to corroborate our findings in humans but to extend our investigations on other tissues. However, our findings are limited by the comparison of metabolic analytes in two different blood matrices and species: human serum and mouse plasma. In theory, the analytical method could be affected by the difference in matrix, and delicate analytes could deteriorate during the prolonged preparation time of serum compared with that of plasma. Therefore, a direct comparison between the matrices serum and plasma has limitations (49). With respect to this, we compared the serum metabolites only within humans, the plasma metabolites only within mice, and each mice tissue separately (50). The observational nature of cohort studies and the applied methods are of purely statistical character, yet still they offer the opportunity to identify unknown coherences and to design study settings to confirm underlying mechanisms. Because of the fact that NO is below the mass cutoff imposed on the instruments, our investigations did not contain measurements of this chemical compound. Nevertheless, our observations suggest further investigations with a specific design to address the involvement of the NO and urea cycle in metformin treatment.

In summary, we observed that serum values of citrulline were reduced under metformin treatment in human patients with T2D and, in a translational approach, also in plasma, skeletal muscle, and epididymal adipose tissue of diabetic mice. The underlying mechanism is most likely the metformin-induced activation of AMPK and its consequent increase of eNOS activity, which is linked to citrulline by the NO cycle.

Acknowledgments. The authors express their appreciation to all KORA study participants for donating their blood and time. The authors thank the field staff in Augsburg, Germany, for conducting the KORA studies. The authors are grateful to the staff (Julia Scarpa, Katharina Faschinger, Franziska Scharl, Nadine Lindemann, Humberto Chavez, and Arsin Sabunchi) from the Institute of Epidemiology at the Helmholtz Zentrum München and the Genome Analysis Center Metabolomics Platform who helped with the sample logistics, data and straw collection, and metabolomic measurements. Additionally, the authors thank the staff (especially Andrea Schneider, Anja Ludolph, Sladjana Jelic, and Birgit Langer) from the Institute of Genetic Epidemiology at the Helmholtz Zentrum München and the platform KORA project application self-service tool (KORA.PASST) for their help with KORA data logistics. The authors also thank the people of the Institute of Diabetes and Regeneration Research (Anett Seelig and Jürgen Schultheiß) and the Institute of Experimental Genetics (Moya Wu), as well as the animal caretaker staff of the German Mouse Clinic, for excellent technical assistance.

Funding. The KORA study was initiated and financed by the Helmholtz Zentrum München German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung) and by the State of Bavaria. Furthermore, KORA research was supported by the Munich Center of Health Sciences (MC-Health), Ludwig-Maximilians-Universität, as part of LMUinnovativ. Part of this project was supported by European Union Seventh Framework Programme grants HEALTH-2009-2.2.1-3/242114 (Project OPTiMiSE) and HEALTH-2013-2.4.2-1/602936 (Project CarTarDis). The German Diabetes Center is funded by the German Federal Ministry of Health (Bundesministerium für Gesundheit, Berlin, Germany) and the Ministry of Innovation, Science and Research of the State of North Rhine-Westphalia (Düsseldorf, Germany). The diabetes part of the KORA F4 study was funded by a grant from the Deutsche Forschungsgemeinschaft (DFG; RA 459/3-1). This study was supported in part by a grant from the Bundesministerium für Bildung und Forschung to the German Center for Diabetes Research (DZD) and by the research consortium "Systems Biology of Metabotypes" (SysMBo grant 0315494A). K.Su. is supported by Biomedical Research Program funds at Weill Cornell Medical College in Qatar, a program funded by the Qatar Foundation.

Duality of Interest. M.F.S. was employed at Helmholtz Zentrum München GmbH during the execution of this study. M.F.S. is currently an employee of the Diabetes Medical Department of AstraZeneca GmbH (Wedel, Germany); however, the company was not involved in work related to data and manuscript generation. R.P.M. is an employee of Metabolon, Inc. S.N. was employed by the Helmholtz Zentrum München GmbH during the execution of this study. S.N. is currently an employee of Sanofi Deutschland GmbH; however, the company was not involved in work related to data and manuscript generation. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. J.Adam, S.B., and R.W.-S. wrote the manuscript. J.L., M.F.S., R.P.M., M.Rot., M.T., S.C., C.H., Y.L., D.A., T.M., M.Rod., and S.N. assisted in manuscript generation. J.Adam, J.L., T.X., and J.B analyzed the data and interpreted the results. M.F.S., R.P.M., M.H., C.H., W.R., G.G., J.Adams., T.I., K.St., C.G., A.P., K.Su., M.H.d.A., S.N., and G.K. performed the experiments, including metabolic profiling. G.K. and R.W.-S. conceived and designed the study. R.W.-S. is the guarantor of this work and, as such, had full access to all 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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