Plasma Metabolomics Identifies Markers of Impaired Renal Function: A Meta-analysis of 3089 Persons with Type 2 Diabetes

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Context: There is a need for novel biomarkers and better understanding of the pathophysiology of diabetic kidney disease.

Objective: To investigate associations between plasma metabolites and kidney function in people with type 2 diabetes (T2D).

Design: 3089 samples from individuals with T2D, collected between 1999 and 2015, from 5 independent Dutch cohort studies were included. Up to 7 years follow-up was available in 1100 individuals from 2 of the cohorts.

Main outcome measures: Plasma metabolites (n = 149) were measured by nuclear magnetic resonance spectroscopy. Associations between metabolites and estimated glomerular filtration rate (eGFR), urinary albumin-to-creatinine ratio (UACR), and eGFR slopes were investigated in each study followed by random effect meta-analysis. Adjustments included traditional cardiovascular risk factors and correction for multiple testing.

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Results: In total, 125 metabolites were significantly associated ($P_{FDR} = 1.5 \times 10^{-32} - 0.046$; β = −11.98-2.17) with eGFR. Inverse associations with eGFR were demonstrated for branched-chain and aromatic amino acids (AAAs), glycoprotein acetyls, triglycerides (TGs), lipids in very low-density lipoproteins (VLDL) subclasses, and fatty acids ($P_{FDR} < 0.03$). We observed positive associations with cholesterol and phospholipids in high-density lipoproteins (HDL) and apolipoprotein A1 ($P_{FDR} < 0.05$). Albeit some metabolites were associated with UACR levels ($P < 0.05$), significance was lost after correction for multiple testing. Tyrosine and HDL-related metabolites were positively associated with eGFR slopes before adjustment for multiple testing ($P_{Tyr} = 0.003$; $P_{HDLrelated} < 0.05$), but not after.

Conclusions: This study identified metabolites associated with impaired kidney function in T2D, implying involvement of lipid and amino acid metabolism in the pathogenesis. Whether these processes precede or are consequences of renal impairment needs further investigation. (***J Clin Endocrinol Metab* 105: 1–13, 2020**)

Key Words: metabolomics, NMR, renal function, albuminuria, meta-analysis

Diabetic kidney disease (DKD) is a frequent complication of diabetes. DKD may lead to end-stage renal disease (ESRD) and is independently associated with a higher risk of all-cause and cardiovascular mortality (1). DKD is often asymptomatic until the very late stages. Therefore, yearly screening of individuals with type 2 diabetes (T2D) with measurement of kidney functions through estimated glomerular filtration rate (eGFR) and urinary albumin-to-creatinine ratio (UACR) is recommended in clinical practice. eGFR and UACR are surrogate markers of DKD (2); however, UACR may not be affected in all individuals with DKD and the decline in eGFR, albeit gradual, is majorly detectable in the later stages of DKD (chronic kidney disease [CKD] stage 3 onward). Moreover, targeted treatment options for DKD are missing and, thus, are currently limited to control of traditional cardiovascular risk factors such as levels of blood pressure, blood lipids, and blood glucose (3). There have been several genome-wide association studies for DKD and kidney function (4-7) suggesting a genetic component. There is an urgent need to identify lifestyle-associated biomarkers for early detection of individuals at a risk for DKD and related metabolic functions (8). Using surrogate quantitative measures for DKD (kidney function decline and albuminuria) may offer greater statistical power and a better understanding of DKD pathophysiology, further leading to discovery of novel treatment targets.

Advances in metabolomics technologies have allowed for a more in-depth characterization of circulating metabolites, thereby adding information simultaneously on multiple metabolic pathways and allowing for a better understanding of the underlying metabolic processes in DKD (9). Additionally, this also adds to the missing lifestyle information required to uncover novel disease mechanisms. Nuclear magnetic resonance (NMR) spectrometry provides a platform for the targeted measurement of amino acids, lipoprotein subclasses, and other metabolites (10,11).

Previous studies involving NMR metabolomics and DKD have mainly been performed in individuals with type 1 diabetes (12-16). Two recent European studies, using NMR metabolomics in T2D, demonstrated that tyrosine is a marker of microvascular complications, but DKD was not investigated separately (17). Second, Barrios et al demonstrated associations of several metabolites, mainly lipids, amino acids, and energy metabolites, with measures of kidney function and incident DKD in 926 persons with T2D and 4838 persons without diabetes (18), although only taking into account a limited number of relevant confounders.

The aim was to investigate associations between plasma metabolites and kidney function in 5 independent Dutch cohorts of individuals all diagnosed with T2D. This study is hypothesis-generating and may identify more metabolic traits of both amino acids and lipids in DKD.

Materials and methods

Participants

In total, 3089 persons with T2D from 5 independent Dutch cohort studies, the Hoorn Diabetes Care System (DCS) West-Friesland (19), the Maastricht study (20), the Rotterdam study (RS) (21), the Netherlands Epidemiology of Obesity (NEO) study (22), and the Cohort of Diabetes and Atherosclerosis Maastricht study (CODAM) (23) were included. The selection processes of the independent studies have previously been described in detail; a brief description of the selection from each cohort for the present study, is provided in the following paragraphs. The studies were all conducted following the Declaration of Helsinki, the local ethics committees approved the original protocols, and all participants gave written informed consent.

The Hoorn Diabetes Care System West-Friesland

The DCS provides diabetes care to people with T2D living in the West-Friesland region, who yearly visit the DCS research center (19). At the yearly visits, a medical exam is performed, and blood is drawn for biochemistry. Individuals are advised on health and treatment and have been invited to participate in the DCS research and biobank (n = 5000+). For the present study, a random sample of individuals from the DCS biobank (n = 750) as well as a selected group of individuals (n = 245) was included, all with available plasma samples collected in 2008-2009. The selected group consisted of individuals with known diabetes complications and individuals who were unable to reach the treatment target of hemoglobin A1c (HbA1c) < 53 mmol/mol. Annual measurements of eGFR were available for calculation of eGFR slopes (median 4 years, interquartile range 2-6 years) in all participants.

The Maastricht Study

The Maastricht Study is a prospective population-based cohort study of individuals aged between 40 and 75 years in the southern part of the Netherlands. Inclusion began in 2010 and is ongoing (20). The cohort is enriched with people with T2D. The study is an in-depth phenotyping study focusing on etiology, complications, and comorbidities of T2D. For the present study, all participants with T2D and available plasma samples (n = 848) were included.

The Rotterdam Study

The Rotterdam Study is a prospective population-based cohort study in the Ommoord district in Rotterdam (21). All inhabitants in the district, aged above 55 years, were invited to participate in this study since 1989, with visits being performed every 3 to 4 years. The plasma samples analyzed for the present study were from RS 1-4 and RS 2-2 cohorts (2002-2005) including people with T2D (n = 426).

The Netherlands Epidemiology of Obesity Study

The NEO study is a prospective population-based cohort study, including individuals aged between 45 and 65 years (n = 6671) from 2008 to 2012, designed for deep phenotyping of pathways leading to obesity-related diseases (22). In the present study, all individuals with T2D at baseline (n = 675) were included.

The cohort of diabetes and atherosclerosis Maastricht study

The CODAM study is a prospective observational cohort study of individuals at increased risk of T2D and cardiovascular disease aged above 40 (n = 574) aiming to investigate the effects of glucose metabolism, lipids, lifestyle, and genetics on (development of) T2D and cardiovascular complications (23). Baseline samples were collected from 1999 to 2002, and eGFR measurements were available from a follow-up visit 7 years after baseline. All individuals with T2D and available plasma samples (n = 145) were included in the present study.

Outcome

The eGFR was calculated from serum creatinine measured locally by the Chronic Kidney Disease Epidemiology Collaboration equation (24). eGFR slope was calculated based on measurements from annual visits in DCS, in participants with at least 2 measurements and a minimum follow-up of 3 years and from the 7-year follow-up visit in CODAM.

UACR was measured in first-morning void spot urine in NEO, CODAM, and DCS. In the Maastricht study, urine albumin excretion (UAE) was based on the average of two 24-hour urine collections. Urine albumin excretion was not measured in the Rotterdam study. People were stratified as having microalbuminuria if UACR was ≥2.5 mg/mmol for men and 3.5 mg/mmol for women. In the Maastricht study, microalbuminuria was present if the UAE was ≥30 mg/d. Macroalbuminuria was defined as UACR ≥ 25 mg/mmol (men) and 35 mg/mmol (women), and in the Maastricht study, as UAE ≥ 300 mg/d.

Standardized methods measured levels of HbA1c, serum/plasma cholesterol, and TG. Brachial blood pressure was measured after at least 5 min rest with an automatic device and an appropriately sized cuff. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Smoking status was defined as yes or no current smoker. Diabetes duration was obtained from medical records or self-reported. Medication use was registered according to Anatomical Therapeutic Chemical classification coding: statins (C10AA, C10BA, C10BX), other lipid-modifying agents (C10AB, C10AC, C10AD, C10AX, C10BA), renin-angiotensin system–blocking agents (angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers; C09), all other antihypertensives (C02, C03, C07, C08), oral glucose lowering drugs (A10B; mainly metformin and sulfonylurea), and insulins (A10).

Metabolic biomarker profiling

The fasted ethylenediamine tetra-acetate plasma samples were stored at −80°C until analysis. The sample storage time varied from 1-15 years. Metabolic biomarkers (n = 149) were quantified from plasma samples of 3089 individuals using high-throughput proton NMR metabolomics (Nightingale Health Ltd, Helsinki, Finland). The method provides concurrent quantification of lipids, 14 lipoprotein subclasses, fatty acid composition, and various low-molecular metabolites, including amino acids, ketone bodies, and gluconeogenesis-related metabolites in molar concentration units (25). The samples do not undergo any extraction steps and the serum samples are never in contact with the NMR detector; thus, there is no significant batch effect in the NMR-based metabolite quantification. Since the preanalytical conditions may vary slightly between different studies, it is recommended to meta-analyze the data as has been done in the present study. Details of the experimentation and applications of the NMR metabolomics platform have been described previously (10,11). Metabolites with equal to or less than 20% missing values were included. After excluding missing data (on metabolomics measures), we performed quantile normalization (using the R functions, "scale" and "quantile") where we added the 10th percentile to the normalized values, on natural log-transformed data (normally distributed).

To check the relatedness between individual metabolite levels found associated with renal function, we performed a sensitivity analysis (pairwise correlation) within the DCS cohort (n = 995).

Statistical analyses

Continuous variables were reported as means ± standard deviation (SD) for normally distributed data, skewed data were reported as median (interquartile range). Categorical variables were presented as total numbers with corresponding percentages. The combined summary for all variables was performed using a weighted arithmetic mean method.

Cross-sectional analyses of each cohort using linear regression were performed to assess associations of single plasma metabolites with 2 continuous outcome variables: eGFR and UACR/UAE. Cross-sectional analyses of each cohort using logistic regression were performed to test associations between single plasma metabolites and the following two categorical outcomes related to deteriorating kidney function: eGFR < 60 mL/min/1.73m^2 and micro- or macroalbuminuria. Longitudinal eGFR measurements were used to calculate annual slopes for 2 of the cohorts (DCS and CODAM). Linear regression analysis was performed to assess associations between metabolites and eGFR slopes. Adjustment of potential confounders included age, sex, use of statins, other lipid-modifying agents, oral glucose-lowering medications, insulins, renin-angiotensin system–blocking agents and other antihypertensives, systolic blood pressure, BMI, smoking, diabetes duration, HbA$_{1c}$, and baseline UACR/UAE or eGFR, where appropriate. In RS, no urine albumin or HbA$_{1c}$ assessment was performed, and no data on diabetes duration was available. This cohort was, therefore, only included in analyses of eGFR and not adjusted for UAER, diabetes duration, or HbA1c. Results from NEO were not adjusted for diabetes duration because of many missing observations. Individual small-sized cohorts ($n < 200$) having a low number of cases (n$_{cases\_eGFR}$ and n$_{cases\_UACR}$ ≤ 10%) while running the logistic regression models were excluded from the meta-analysis (25). Also, individuals with missing covariate data were excluded from each analysis.

A random effects meta-analysis of the respective study sets was performed using the R meta-package (Meta v4.8-4) for cross-sectional and longitudinal data (eGFR slopes). We compared the results from the meta-analyses of 4 cohorts in the logistic models and of 5 cohorts in the linear models with the results from the meta-analyses including only the 3 cohorts with all covariates available (DCS, Maastricht study, and CODAM). For this analyses, a meta regression model in the R package called *metaphor* (26) was applied. We used a fixed effects model because the (residual) heterogeneity within each subset has already been accounted for by fitting random effects models (27). The fixed effects model did not substantially change the results ($P > 0.05$) (25), and therefore data from the meta-analyses including data from all 5 cohorts are presented, unless stated otherwise. Further, sensitivity analyses were performed in the largest cohort (the DCS cohort) (i) excluding persons aged above 75 years and (ii) including only individuals with eGFR ≥ 60 mL/min/1.73m^2.

Correction for multiple testing was performed by the false-discovery rate (FDR) method (28). A 2-tailed FDR-adjusted P-value (P_{FDR}) < 0.05 was considered statistically significant. Data analysis was performed with R-Studio v1.0.143.

For sensitivity analysis pairwise correlation coefficients (r) using Pearson's method were estimated for the scaled, nonmissing metabolite levels associating with eGFR ($n = 125$). These measures (r) were plotted as a heatmap using the "heatmap.2" function in the statistical R package "gplots." The dendrogram (hierarchical clustering) based on correlation as a distance measure and the metabolite groupings ($n = 14$) were also added to the correlation heatmap.

Results

In the combined populations, 59% of the individuals were men, the mean ± SD age was 64 ± 8 years, and the mean eGFR was 82 ± 16 mL/min/1.73m^2. Clinical characteristics for each participating cohort and their combined summary are presented in Table 1. The largest differences between cohorts were observed in means of age, systolic blood pressure, diabetes duration, and use of different classes of medications. Heterogeneity between cohorts on baseline characteristics was accounted for using random effects meta-analysis. Overall, at baseline, 332 individuals had microalbuminuria, 40 had macroalbuminuria, 293 had eGFR 30–60 mL/min/1.73 m^2, and 7 had eGFR ≤ 30 mL/min/1.73 m^2. The eGFR slopes were based on a median of 4 measurements in 994 participants in DCS and 2 measurements in 106 participants in CODAM, respectively. The mean percentage of missing metabolite values in each of the cohorts was between 0.1% and 0.6% (range 0%-19%).

Cross-sectional associations between metabolites and eGFR

As a continuous measure, eGFR was significantly associated with 125 metabolites (Fig. 1, Table 2) (25). The AAAs phenylalanine (β = –3.05, P_{FDR} = 1.5 × 10^{-32}, P_{het} = 0.99), and histidine (β = –1.11, P_{FDR} = 3.6 × 10^{-5}, P_{het} = 0.5), the branched-chain amino acid (BCAAs) isoleucine (β = –1.92, P_{FDR} = 3.8 × 10^{-10}, P_{het} = 0.49) and leucine (β = –1.16, P_{FDR} = 1.4 × 10^{-3}, P_{het} = 0.22), and the nonessential amino acid glutamine (β = –1.54, P_{FDR} = 8.2 × 10^{-10}, P_{het} = 0.60) were strongly and inversely associated with eGFR, while the AAA tyrosine was positively associated with eGFR. The glycolysis-related metabolites glucose and lactate were also positively associated with eGFR, while citrate and glycoprotein acetyls were inversely associated with eGFR (P_{FDR} < 0.001). Inverse associations with eGFR were observed for acetoacetate, measures of fatty acids, TGs in all lipoprotein subclasses, cholesterols, phospholipids in and particle concentrations of VLDL, intermediate-density lipoprotein, low-density lipoproteins (LDL), sphingomyelins, and apolipoprotein B (P_{FDR} < 0.05). Cholesterol and phospholipids in and particle concentrations of HDL subclasses, HDL particle size, and apolipoprotein A1 were positively associated with eGFR (P_{FDR} < 0.05).

Table 1. Baseline characteristics of the five study cohorts of people with T2D (n = 3089)

	DCS (n = 995)	Maastricht (n = 848)	RS (n = 426)	NEO (n = 675)	CODAM (n = 145)	Combined (n = 3089)
Age, years	63 (10)	63 (8)	76 (6)	58 (5)	61 (6)	64 (8)
Sex: men, n (%)	569 (57)	580 (68)	204 (48)	376 (56)	95 (66)	1824 (59)
Diabetes duration, years	5.9 [2.9–9.1]	6.0 [2.0–10.0]	NA	4.6 [0–7]	0.0 [0.0–5.8]	5.2
Body mass index, kg/m²	30.6 (5.5)	29.9 (4.9)	29.1 (4.5)	33.0 (5.3)	30.3 (4.6)	30.7 (5.1)
SBP, mmHg	143 (19)	142 (18)	156 (21)	137 (17)	148 (19)	143 (19)
HbA$_{1c}$, mmol/mol	48 [43–55]	50 [45–56]	NA	49 [40–55]	50 [44–57]	48.9
HbA$_{1c}$, %	6.5 [6.1–7.2]	6.7 [6.3–7.3]	NA	6.6 [5.8–7.0]	6.7 [6.1–7.3]	6.6
eGFR, mL/min/1.73m²	81 (18)	83 (16)	73 (14)	88 (14)	92 (14)	82 (16)
UACR, mg/mmol	0.6 [0.4–1.3]	NA	NA	0.6 [0.3–1.0]	0.5 [0.3–1.1]	0.6
UAE, mg/d	NA	10 [5–22]	NA	NA	NA	-
Total cholesterol, mmol/L	4.7 (1.9)	4.4 (1.0)	5.3 (1.0)	5.0 (1.2)	5.2 (1.2)	4.7 (1.3)
LDL cholesterol, mmol/L	2.6 (0.9)	2.4 (0.9)	3.6 (0.9)	3.0 (1.1)	3.2 (0.9)	2.7 (0.9)
HDL cholesterol, mmol/L	1.2 (0.4)	1.3 (0.4)	1.3 (0.4)	1.2 (0.3)	1.1 (0.3)	1.2 (0.3)
Triglycerides, mmol/L	1.6 [1.2–2.2]	1.5 [1.1–2.1]	1.5 [1.2–2.1]	1.6 [1.2–2.2]	1.8 [1.2–2.4]	1.56
Smoking, n (%)	167 (17)	128 (15)	59 (14)	120 (18)	26 (18)	500 (16)
History of CVD[a], n (%)	165 (18)	219 (26)	100 (24)	78 (12)	25 (17)	587 (19)
Medication						
Statins, n (%)	685 (69)	627 (74)	123 (29)	355 (53)	35 (24)	1825 (59)
Other lipid-modifying agents, n (%)	31 (3)	41 (5)	8 (2)	4 (1)	3 (2)	87 (3)
RAS-blocking agents, n (%)	498 (50)	499 (59)	152 (36)	313 (46)	42 (29)	1504 (49)
Other antihypertensives, n (%)	568 (57)	428 (50)	267 (63)	424 (63)	69 (48)	1756 (57)
Oral glucose lowering drugs, n (%)	824 (83)	623 (73)	195 (46)	330 (49)	64 (44)	2036 (66)
Insulin use, n (%)	263 (26)	175 (21)	47 (11)	87 (13)	13 (9)	585 (19)
Categorical outcomes						
eGFR 30-60 mL/min/1.73m², n (%)	120 (12)	69 (8)	76 (18)	22 (3)	6 (4)	293 (9)
eGFR ≤ 30 ml/min/1.73m², n (%)	4 (<1)	1 (<1)	1 (<1)	1 (<1)	0	7 (0.5)
Microalbuminuria, n (%)	118 (12)	149 (18)	NA	53 (8)	12 (8)	332 (11)
Macroalbuminuria, n (%)	18 (2)	10 (1)	NA	10 (2)	2 (1)	40 (1)

Mean (SD) or median [interquartile range] for continuous variables, n (%, rounded) for categorical variables.

Abbreviations: CODAM, The Cohort of Diabetes and Atherosclerosis Maastricht study; CVD, cardiovascular disease; DCS, The Hoorn Diabetes Care System; eGFR, estimated glomerular filtration rate; NEO, The Netherlands Epidemiology of Obesity Study; RAS, renin-angiotensin system; RS, Rotterdam Study; SBP, systolic blood pressure; UACR, urinary albumin-to-creatinine ratio; UAE, urinary albumin excretion.

[a]Self-reported.
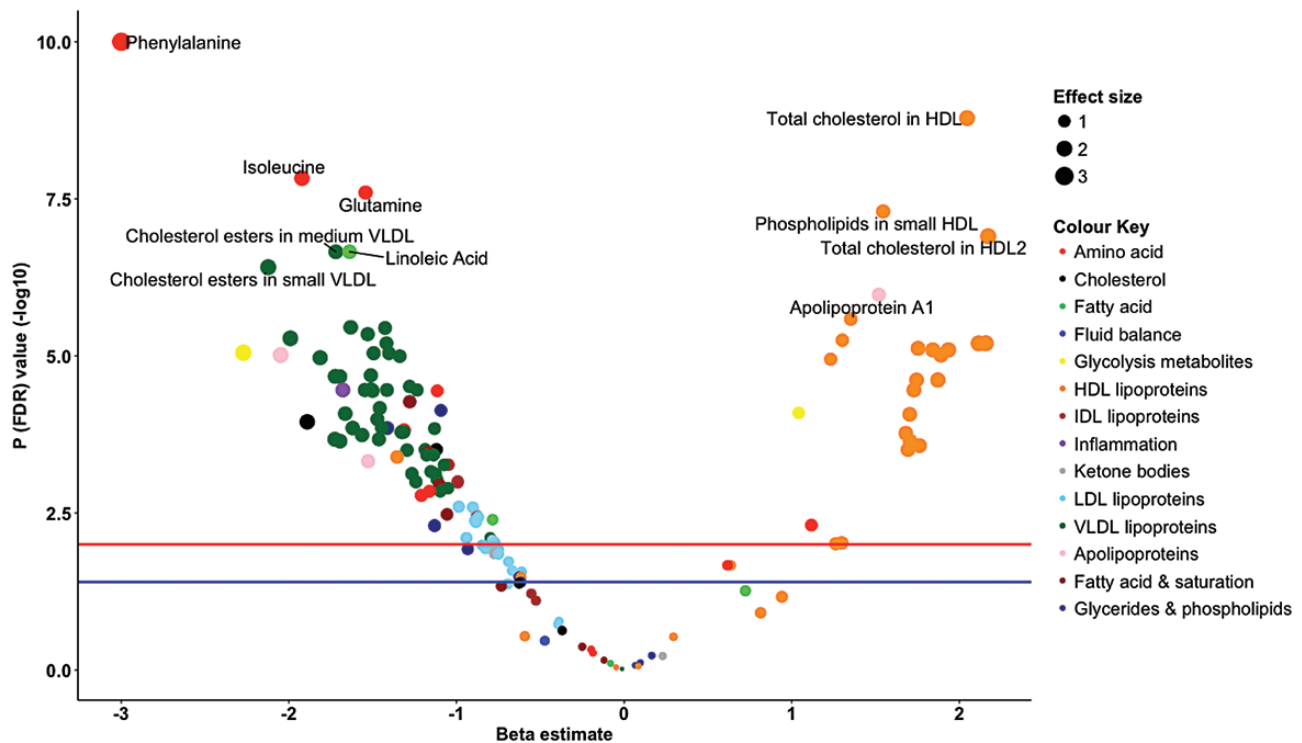
Figure 1. Volcano plot of eGFR and associated metabolites. The Y axis represents $-\log_{10}$ (P_{FDR} value) for metabolite eGFR association. Blue line represents P-value = 0.05, and red line, P_{FDR}-value = 0.05. Metabolites are mmol/L, log transformed. P_{FDR} values < 1.0×10^{-10} are depicted as 1.0×10^{-10}, beta estimates ≥−3.0 are depicted as −3.0 to fit into the figure. Top 10 most significant metabolites are named. Each dot signifies 1 metabolite, and the size of the dot relates to the observed estimates from the random effects meta-analysis. Color of the dot determines the metabolite group listed under the color key.

In the logistic regression analyses of having a low eGFR < 60 mL/min/1.73 m^2, significant results were demonstrated for 106 metabolite measures, of which 104 overlapped with those significant in the linear regression analyses (25). Phenylalanine was the strongest signal associated with the maximum likelihood of having low eGFR in the logistic regression model (odds ratio = 1.67, P_{FDR} = 4.1×10^{-13}, P_{het} = 0.38).

Cross-sectional associations between metabolites and albuminuria

UAER/UAE level was associated with 11 metabolites ($P < 0.05$) (Fig. 2) of which 9 were also associated with eGFR. Positive associations with glucose, glycoprotein acetyls, phosphatidylcholine, and 2 lipid measures in VLDL subclasses were demonstrated. Citrate, glutamine, and (free) cholesterol and phospholipids in very large HDL were negatively associated with UACR. Significance for all tested associations was lost after correction for multiple testing (25). In the logistic regression analyses, albuminuria (micro- or macroalbuminuria) was associated with 22 metabolite measures ($P < 0.05$) of which 18 were also associated with eGFR. Tyrosine was inversely associated with albuminuria while glucose; glycoprotein acetyls; phosphoglycerides; phosphatidylcholine; apolipoprotein B; content of TGs in

lipoproteins, total, free, and VLDL cholesterol; some lipids in (very) small VLDL; and several fatty acids were positively associated with albuminuria. However, after adjustment for multiple testing, the results were no longer significant (25). In the Maastricht study, where albuminuria was measured in 24-h urine samples, we observed minor changes to βs and P-values when adjusting for eGFR. Moreover, the heterogeneity between studies was small.

Associations between metabolites and eGFR slope

Eleven metabolites were associated with eGFR slopes ($P < 0.05$) before adjustment for multiple testing. Tyrosine and HDL related metabolites were positively associated with eGFR slopes and thereby demonstrated the same directionality as in the cross-sectional analyses. However, after adjustment for multiple testing, the results were no longer significant (25).

Sensitivity analyses

Sensitivity analyses, including only the DCS cohort and excluding individuals aged above 75 years ($n = 163$), did not substantially affect the results. This result suggests that the observed associations were not driven by age. We also did not observe significant differences in the metabolites most significantly associated with eGFR

Table 2. Metabolites Associated with eGFR (n = 3079)—Divided by Metabolite Subgroups

Metabolites	β	SE	P	P_{FDR}	P_{het}
Amino acids					
Phenylalanine	−3.05	0.25	2.0×10^{-34}	1.5×10^{-32}	0.99
Isoleucine	−1.92	0.31	3.9×10^{-10}	1.5×10^{-8}	0.30
Glutamine	−1.54	0.25	8.2×10^{-10}	2.5×10^{-8}	0.49
Histidine	−1.11	0.25	1.0×10^{-5}	3.6×10^{-5}	0.50
Leucine	−1.16	0.35	8.4×10^{-4}	1.4×10^{-3}	0.22
Tyrosine	0.62	0.26	1.7×10^{-2}	2.1×10^{-2}	0.68
Inflammation					
Glycoprotein acetyls, mainly a1-acid glycoprotein	−1.68	0.38	8.9×10^{-6}	3.5×10^{-5}	0.10
Glycolysis related metabolites					
Citrate	−2.27	0.47	1.4×10^{-6}	8.9×10^{-6}	0.02
Lactate	1.04	0.25	2.6×10^{-5}	8.1×10^{-5}	0.47
Glucose	1.12	0.38	3.0×10^{-3}	5.0×10^{-3}	0.99
Ketone bodies					
Acetoacetate	−0.77	0.30	1.0×10^{-2}	1.0×10^{-2}	0.99
Fatty acids					
18:2 Linoleic acid	−1.64	0.29	1.3×10^{-8}	2.2×10^{-7}	0.37
Omega-6 fatty acids	−1.28	0.30	1.6×10^{-5}	5.4×10^{-5}	0.33
Polyunsaturated fatty acids	−1.17	0.31	1.6×10^{-4}	3.4×10^{-4}	0.29
Monounsaturated fatty acids; 16:1, 18:1	−1.10	0.32	6.4×10^{-4}	1.1×10^{-3}	0.21
Total fatty acids	−1.06	0.34	2.0×10^{-3}	3.3×10^{-3}	0.19
Saturated fatty acids	−0.73	0.35	3.9×10^{-2}	4.6×10^{-2}	0.16
Lipoprotein particle sizes					
HDL_D	1.74	0.38	5.7×10^{-6}	2.4×10^{-5}	0.11
VLDL_D	−1.07	0.30	3.0×10^{-4}	5.0×10^{-4}	0.29
Cholesterol					
Total cholesterol in HDL	2.05	0.31	3.2×10^{-11}	1.6×10^{-9}	0.29
Total cholesterol in HDL2	2.17	0.37	5.7×10^{-9}	1.3×10^{-7}	0.13
Total cholesterol in VLDL	−1.81	0.38	2.1×10^{-6}	1.1×10^{-5}	0.10
Remnant cholesterol (non-HDL, non-LDL -cholesterol)	−1.89	0.46	3.9×10^{-5}	1.1×10^{-4}	0.19
Free cholesterol	−1.12	0.29	1.4×10^{-4}	3.1×10^{-4}	0.78
Total cholesterol in IDL	−0.88	0.29	2.2×10^{-3}	3.7×10^{-3}	0.52
Total cholesterol in LDL	−0.80	0.29	5.3×10^{-3}	7.8×10^{-3}	0.43
Serum total cholesterol	−0.63	0.30	3.4×10^{-2}	4.1×10^{-2}	0.59
Apolipoproteins					
Apolipoprotein A-1	1.52	0.28	7.6×10^{-8}	1.1×10^{-6}	0.87
Apolipoprotein B	−1.53	0.42	2.3×10^{-4}	4.7×10^{-4}	0.07
Glycerides and phospholipids					
Triglycerides in HDL	−1.10	0.26	2.3×10^{-5}	7.4×10^{-5}	0.54
Serum total triglycerides	−1.41	0.35	5.1×10^{-5}	1.4×10^{-4}	0.15
Triglycerides in VLDL	−1.44	0.36	5.1×10^{-5}	1.4×10^{-4}	0.13
Diacylglycerol	−0.93	0.36	8.8×10^{-3}	1.2×10^{-2}	0.15
Sphingomyelins	−0.63	0.28	2.7×10^{-2}	3.3×10^{-2}	0.55
Triglycerides in LDL	−0.61	0.29	3.2×10^{-2}	4.0×10^{-2}	0.34
Lipoprotein subclasses of VLDL[a]					
Cholesterol esters in medium VLDL	−1.72	0.30	1.3×10^{-8}	2.2×10^{-7}	0.27
Cholesterol esters in small VLDL	−2.12	0.38	2.5×10^{-8}	3.9×10^{-7}	0.12
Total cholesterol in medium VLDL	−1.63	0.32	3.0×10^{-7}	3.5×10^{-6}	0.22
Lipoprotein subclasses of HDL[a]					
Phospholipids in small HDL	1.55	0.26	2.0×10^{-9}	5.0×10^{-8}	0.61
Free cholesterol in small HDL	1.35	0.26	2.0×10^{-7}	2.6×10^{-6}	0.90
Total lipids in small HDL	1.30	0.26	6.3×10^{-7}	5.6×10^{-6}	0.82
Lipoprotein subclasses of IDL[a]					
Cholesterol esters in IDL	−0.99	0.29	5.5×10^{-4}	1.0×10^{-3}	0.43
Total lipids in IDL	−0.99	0.29	5.8×10^{-4}	1.0×10^{-3}	0.48
Total cholesterol in IDL	−0.87	0.29	2.3×10^{-3}	3.7×10^{-3}	0.52
Lipoprotein subclasses of LDL[a]					
Triglycerides in small LDL	−0.99	0.31	1.5×10^{-3}	2.5×10^{-3}	0.25
Cholesterol esters in medium LDL	−0.90	0.29	1.6×10^{-3}	2.5×10^{-3}	0.40
Cholesterol esters in large LDL	−0.88	0.29	2.1×10^{-3}	3.3×10^{-3}	0.41

Metabolites are mmol/L except apolipoproteins, which are g/L. All metabolites are log transformed. P_{het}-value for heterogeneity. Adjustment of potential confounders included age, sex, use of statins, other lipid-modifying agents, oral glucose lowering medications, insulins, RAS-blocking agents and other antihypertensives, SBP, BMI, smoking, diabetes duration, HbA1c, and baseline UACR/UAE. In RS, no urine albumin or HbA1c assessment
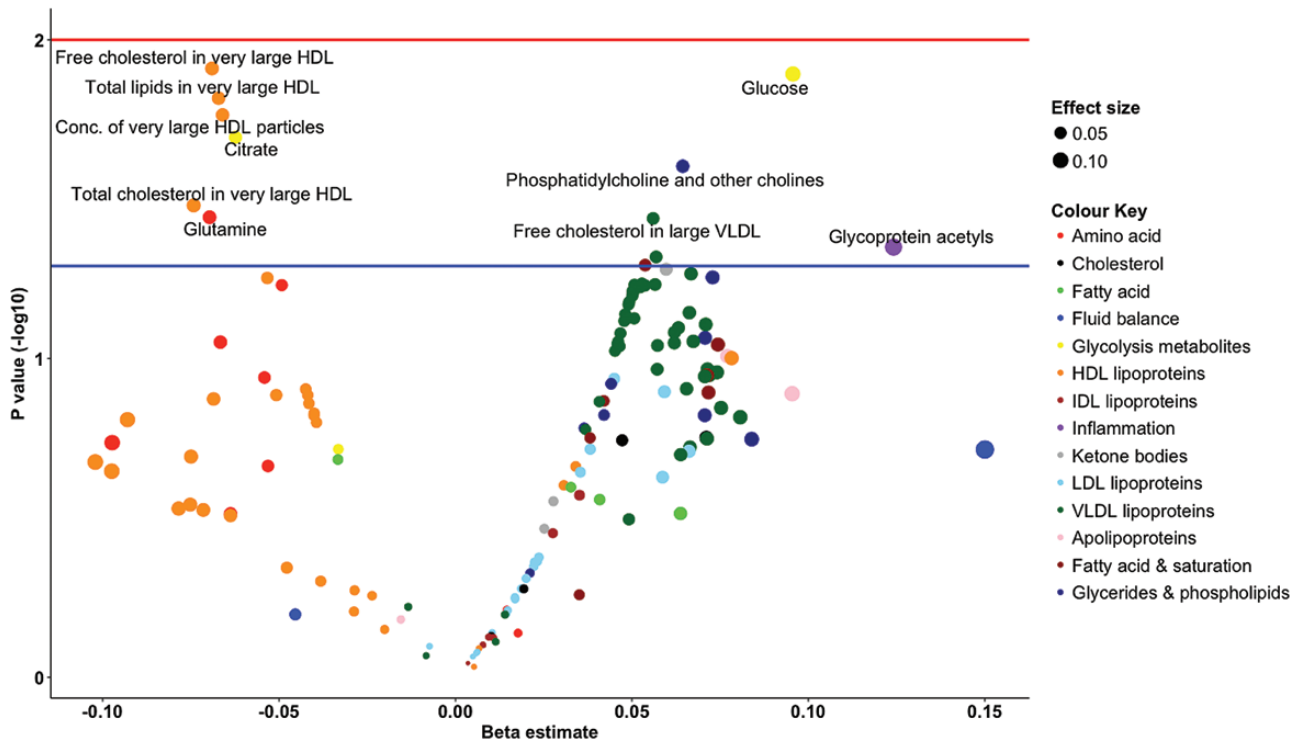
Figure 2. Volcano plot of UAER/UAE and associated metabolites. The Y axis represents $-\log_{10}$ (P-value) for metabolite albuminuria (UAER/UAE) association. Blue line represents P-value = 0.05, and red line, P_{FDR} value = 0.05. Metabolites are mmol/L, log transformed. P_{FDR} values < 1.0×10^{-10} are depicted as 1.0×10^{-10}. β estimates ≥0.15 are depicted as 0.15. Top 10 most significant metabolites have been named. Each dot signifies 1 metabolite and the size of the dot relates to the observed estimates from the random effects meta-analysis. Color of the dot determines the metabolite group listed under the color key.

after omission of people with eGFR < 60 mL/min/m^2 (n = 237).

It is known that eGFR tends to increase initially and then decrease during DKD progression. Therefore we performed another sensitivity analysis within the DCS cohort where we compared metabolite associations with eGFR tertiles. We observed 74% consistency in the directionality of effects across tertiles (2 out of 3). Consistency of 100% could not be achieved due to a lack of statistical power for this nested analysis in the DCS cohort (data not shown).

To account for the relatedness between different metabolite levels associated with eGFR/UACR, a heatmap depicts the correlation between individual metabolites and metabolite groups within the DCS cohort (n = 995) (Fig. 3). The lipoprotein groups indicate that the metabolites are not entirely independent. A general trend shows highly correlated metabolites within a specific lipoprotein groups (VLDL lipoproteins, and HDL lipoproteins) while also highlighting intergroup differences (positive [blue] vs negative [red] correlations) (Fig. 3).

Discussion

In this study, we investigated associations between plasma metabolites and kidney function (cross-sectionally and longitudinally) in individuals with T2D. The key findings include several novel associations between eGFR and circulating amino acids, TGs, lipids in VLDL subclasses, free fatty acids, and lipids in HDL subclasses cross-sectionally. eGFR slopes associated with tyrosine and subclasses of HDL lipoproteins (before correction for multiple testing). None of the metabolites measured were significantly associated with urinary albumin excretion. These findings are in line with results from a recent study in type 1 diabetes where metabolites were mainly associated with eGFR and not albuminuria (29).

The AAA phenylalanine was strongest inversely associated with eGFR levels, followed by the BCAA isoleucine and the polar amino acid glutamine. This result replicates findings from a recent study by Barrios et al, where a strong inverse cross-sectional association between phenylalanine and eGFR was demonstrated both in 926 individuals with T2D and 4838 individuals
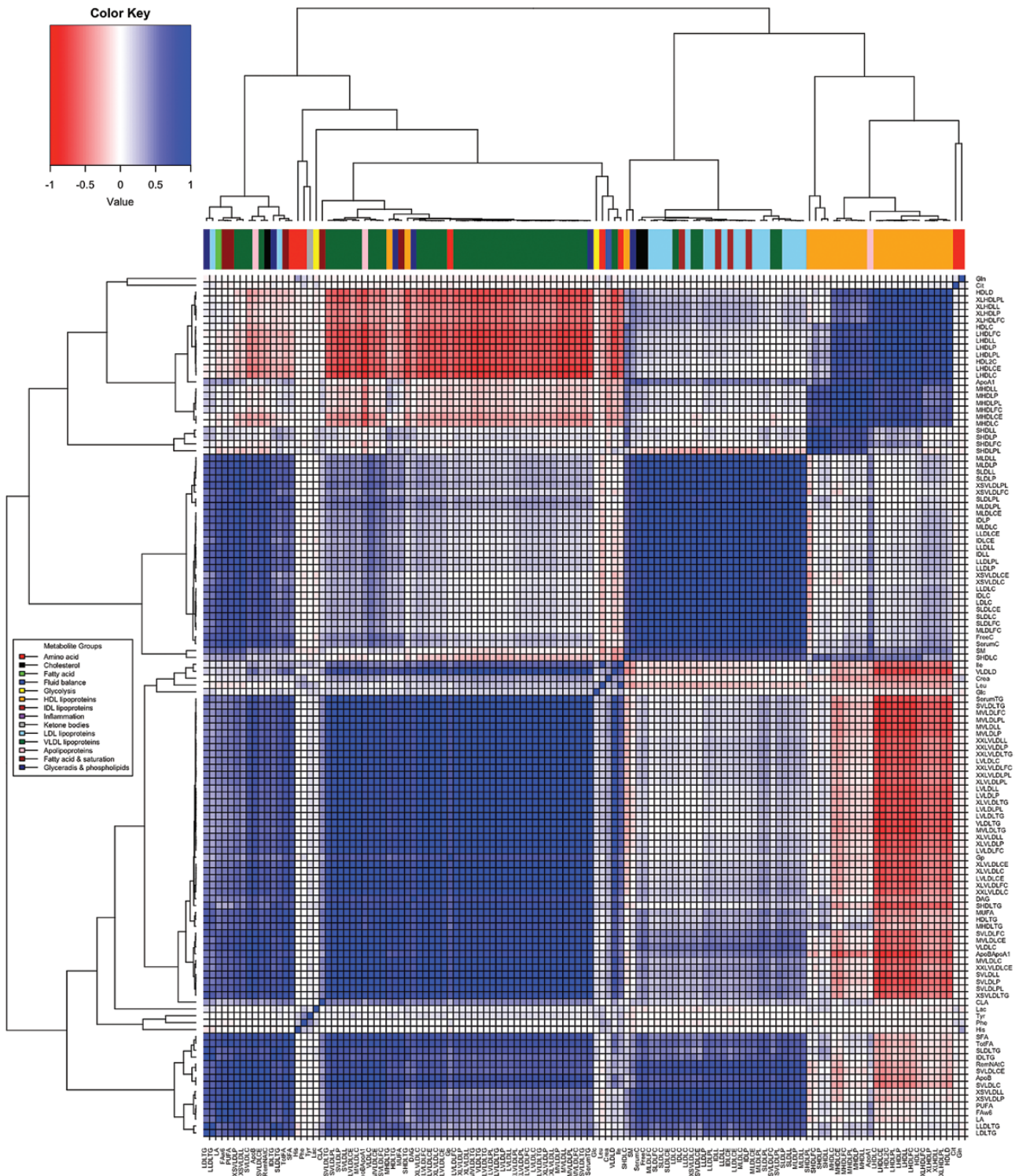
Figure 3. Correlation heatmap of individual metabolites, metabolite groups, and hierarchical clustering. eGFR-associated metabolite levels (*n* = 125) within the DCS cohort are plotted (quantile normalized). Each small square on the X and Y axis depicts individual metabolites. Higher color intensity (blue/red) indicates high values of positive/negative pairwise correlation measure between metabolites. Dendrogram depict clustering based on correlation distance measures. Horizontal colored bar on top of the heatmap depicts the metabolite groups.

without diabetes, after adjusting for age, sex, BMI, statin use, and hormone replacement therapy (18). Barrios et al did not find an association between phenylalanine and longitudinal changes in eGFR, similar to the findings in this study. Higher phenylalanine and lower tyrosine

levels due to impaired renal conversion of phenylalanine have previously been reported in renal disease (30,31). In our study, a positive association between tyrosine and eGFR was also observed cross-sectionally and longitudinally. Recently, the results of ADVANCE trial in 3587

individuals with T2D demonstrated a positive association of phenylalanine with macrovascular diseases and all-cause mortality; however, this was attenuated after adjustment for cardiovascular risk factors. Moreover, this study showed an inverse association between tyrosine and microvascular complications (17). This trial demonstrated that higher levels of aromatic (histidine) and branched-chain (leucine) amino acids were associated with a lower risk of all-cause mortality. This is in line with our findings in which histidine and leucine were inversely associated with eGFR. In a study by Niewczas et al comparing T2D progressors (*n* = 40) to ESRD and nonprogressors (*n* = 40), higher levels of phenylalanine, tyrosine, and leucine were associated with a lower risk of progression to ESRD during 12 years follow-up, albeit the results were not statistically significant after adjustment for HbA1c, albumin excretion, eGFR, and multiple testing (32). The population had a longer diabetes duration compared to this study, and one may speculate that a different metabolite profile could be observed in the early courses of T2D and/or DKD. Unfortunately, we were limited to examining the longitudinal associations with the eGFR slope and did not have data on ESRD. On the other hand, our study is statistically well-powered compared to the study by Niewczas et al. Moreover, methodological differences between the current (NMR based) and the previously discussed studies (mass-spectrometry [MS] based) might explain some of the discrepancies.

In this study, isoleucine and leucine were inversely linearly associated with eGFR, and in line with this, higher isoleucine levels were also associated with an increased likelihood of decreased kidney function in the logistic model. In a previous paper from the same 5 cohorts, higher levels of isoleucine and leucine were associated with a higher risk of having an HbA1c >53 mmol/L as a measure of dysregulated diabetes (33). A link between BCAAs and the development of diabetes (34), as well as insulin resistance (35,36), has previously been demonstrated. This suggests that BCAAs (isoleucine and leucine) levels may be indicative of not only dysregulated diabetes and insulin resistance but also progression to DKD. Indeed, insulin resistance has previously been hypothesized to play an essential role in DKD (37), especially in individuals with T2D. Several mechanisms could potentially explain the increased levels of BCAAs in individuals with insulin resistance. For instance, in individuals without diabetes, it has been proposed that high BCAA levels originate from the gut microbiome (38). Exploring this further in individuals with T2D could shed further light on the pathophysiology and potentially reveal treatment targets.

We demonstrated in the current study an inverse association between eGFR and glutamine in linear regression analyses. This inverse relationship is in line with the previous study by Niewczas et al, where higher glutamine was associated with a higher risk of ESRD, although not statistically significant after adjustment for HbA1c, albumin excretion, eGFR, and multiple testing (32). In contrast, we previously reported that higher glutamine was most significantly associated with having an HbA1c < 53 mmol/L (33).

In this study, eGFR was inversely associated with TGs, lipid measures in VLDL subclasses, sphingomyelin, and fatty acids like omega-6 and linoleic acid but positively associated with lipid measures, except TGs, in HDL subclasses. This inverse relationship is in line with results in both individuals with and without diabetes from the previously mentioned study by Barrios et al (18). The associations between these lipid measures and longitudinal endpoints of ESRD and macro- or microvascular complications have to our knowledge not been tested in any study in people with T2D. We identified the lipids in HDL to be associated positively with longitudinal eGFR slopes among T2D, which became nonsignificant after FDR correction. These could be correct signals reflecting cross-sectional results on eGFR in our study. The longitudinal results in our study may have missed the multiple testing threshold due to lower statistical power (*n* = 1100) compared to cross-sectional meta data where we had 3089 individuals. On the other hand, we cannot undermine the possibility of intermetabolite relatedness as suggested by the sensitivity analysis (correlation heatmap) that may reduce the number of tests. However, in a longitudinal study by the FinnDiane Study Group including 3544 individuals with type 1 diabetes, the TG–cholesterol imbalance was associated with progression in albuminuria and all-cause mortality, as was a large HDL cholesterol (14). Previous studies in large cohorts of dyslipidemia and DKD have demonstrated associations between routinely measured higher TG and lower HDL cholesterol levels to the progression of DKD in individuals with T2D (39,40). Higher TG and lower HDL cholesterol are clinical components of the metabolic syndrome and may also be consequences of the underlying insulin resistance (41). Targeting HDL cholesterol or phospholipids may be a future therapeutic approach, although previous clinical studies with HDL cholesterol increasing agents—for example, torcetrapib (42) in high-risk individuals of cardiovascular disease—have demonstrated increased risk of mortality and morbidity of unknown mechanism. In a small study by Drew et al (*n* = 13), intravenous reconstituted HDL infusion in patients with T2D increased plasma insulin. It activated

adenosine 5′-monophosphates–activated protein kinase in skeletal muscle when compared to placebo (43).

The major strengths of this study are the large number of individuals with T2D from 5 independent cohorts as well as adjustment for an extensive set of relevant clinical covariates and multiple testing, which diminished the risk of false-positive results. Moreover, the use of the NMR platform provided standardized measures of metabolites, allowing exploration of measures beyond routinely measured biomarkers. This method was faster, cheaper, and thereby more accessible than the more extensive MS-based metabolomics methods and could potentially be easier to adapt to a clinical setting, although it did not provide as in-depth a characterization as, for example, MS. The use of medications agents may affect the metabolome substantially. In a recently published paper, also from the Biobanking and Biomolecular Resources Research Infrastructure consortium, significant associations between cardiometabolic agents and several of the measured metabolites were demonstrated (44). In the present study, results were adjusted for several medications commonly used in diabetes. We further recognize some limitations of the study. Metabolites were measured at one timepoint and therefore did not provide information regarding within-subject variation in metabolite levels. In some of the original studies, information regarding albuminuria measurements and history of micro- or macroalbuminuria was very limited. Further, we were limited by a low number of cases in the CODAM study, no diabetes duration in the RS study, and only having follow-up measurements from 2 cohorts.

Information regarding diet, which may affect the measured metabolites, was not available in all cohorts and not uniformly captured. Therefore, we could not control for diet in this study. Information regarding other renal markers, such as vitamin D, parathyroid hormone, and calcium, was also not available. Since the samples originate from different studies, the storage time before metabolite measurements differed. Although all samples were stored at –80°C as recommended (45), we did find consistent results across studies, regardless of the year of sampling. This finding was also demonstrated by low heterogeneity between studies, suggesting no such influence.

In conclusion, the current largest-to-date study identifies metabolites associated with an impaired eGFR among individuals with T2D while none of the metabolites measured were significantly associated with urinary albumin excretion. These results suggest that cholesterol and phosphoglycerides in HDL subclasses may be associated with a better kidney function while high levels of several amino acids and fatty acids, lipids in VLDL subclasses, and TGs in all lipoproteins are associated with an impaired kidney function. The findings suggest alterations in the metabolome associated with renal impairment in T2D of primary importance in nonalbuminuric DKD or the early stages of disease before development of albuminuria. Further longitudinal studies are needed to clarify whether alterations in metabolite levels precede or are consequences of renal impairment and whether a biomarker panel of both amino acid and lipid measures could potentially lead to improved prediction in the development of DKD.

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Disclosure Summary. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed. Dennis Mook-Kanamori works as a part-time clinical research consultant for Metabolon, Inc. All other authors declare no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

Data Availability: The data sets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding authors on reasonable request.

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