Circulating Metabolites and Lipids Are Associated to Diabetic Retinopathy in Individuals With Type 1 Diabetes

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Omics-based methods may provide new markers associated to diabetic retinopathy (DR). We investigated a wide omics panel of metabolites and lipids related to DR in type 1 diabetes. Metabolomic analyses were performed using two-dimensional gas chromatography with time-of-flight mass spectrometry and lipidomic analyses using an ultra-high-performance liquid chromatography quadruple time-of-flight mass spectrometry method in 648 individuals with type 1 diabetes. Subjects were subdivided into no DR, mild nonproliferative DR (NPDR), moderate NPDR, proliferative DR, and proliferative DR with fibrosis. End points were any progression of DR, onset of DR, and progression from mild to severe DR tracked from standard ambulatory care and investigated using Cox models. The cohort consisted of 648 participants aged a mean of 54.4 ± 12.8 years, 55.5% were men, and follow-up was 5.1–5.5 years. Cross-sectionally, 2,4-dihydroxybutyric acid (DHBA), 3,4-DHBA, ribonic acid, ribitol, and the triglycerides 50:1 and 50:2 significantly correlated ($P < 0.042$) to DR stage. Longitudinally, higher 3,4-DHBA was a risk marker for progression of DR ($n = 133$) after adjustment ($P = 0.033$). We demonstrated multiple metabolites being positively correlated to a higher grade of DR in type 1 diabetes and several triglycerides being negatively correlated. Furthermore, higher 3,4-DHBA was an independent risk marker for progression of DR; however, confirmation is required.

One of the most frequent and debilitating complications of diabetes is diabetic retinopathy (DR). Despite radical improvements in diagnosis and treatment throughout the last decades (1), DR is still the primary cause of blindness in individuals with diabetes aged 20–74 years, and the prevalence was 34.6% in a large meta-analysis including 22,896 individuals with diabetes (2). The advances in diagnosis and treatment have particularly been made for later stages of the disease, and robust and specific risk markers for onset and early progression of DR are still lacking.

Novel omics methods have been developed, allowing for simultaneous evaluation of large panels of metabolites using mass spectrometry–based approaches that allow for comprehensive study of metabolic pathways compared with more traditional single-biomarker approaches. Omics facilitates advanced and detailed analysis faster than standard methods. More specifically, metabolomics and lipidomics is the analysis and categorization of circulating metabolites and lipids using this method and can provide a more comprehensive view of the biological effects of various metabolites (3,4).

Applying this method to DR is an intriguing concept. Omics allows for a unique ability to understand the biological pathways for DR as well as facilitate the discovery of novel biomarkers associated to DR development and progression. Few studies have evaluated the association between circulating metabolites and the presence of DR. In one cross-sectional study, several metabolites were associated with DR in type 2 diabetes, among them, hydroxyl fatty acids, such as 3,4 dihydroxybutyric acid (3,4-DHBA), and sugar derivatives such as lactose, maltose, and ribose (5). Likewise, other studies have identified plasma metabolites associated to arginine-, pyrimidine- and fatty acid-related pathways, as well as several amino acids highly related to insulin resistance, in relation to DR (6–8).

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In the current study, we investigated the predictive qualities of a wide panel of metabolites and lipids in plasma in relation to the presence, onset, and progression of DR in individuals with type 1 diabetes (T1D).

RESEARCH DESIGN AND METHODS

Study Population

Between 2009 and 2011, 648 individuals with T1D and a large range of albuminuria were recruited from the outpatient clinic at Steno Diabetes Center Copenhagen. The details of the cohort have previously been described (9). Participants were subdivided by stages of albuminuria (normo-, micro-, and macroalbuminuria). End-stage kidney disease, defined as receiving dialysis, renal transplantation, or an estimated glomerular filtration rate (eGFR) $<$15 mL/min/1.73 m^2 at baseline was an exclusion criterion. In the current study, metabolomics and lipidomics data along with information on retinopathy status was available for 601 (serum metabolomics) and 648 (plasma lipidomics) participants, respectively.

The study was conducted in compliance with the Declaration of Helsinki and was approved by the ethics committee for the Capital Region of Denmark (Hillerød, Denmark). All participants have given informed written consent.

Baseline Clinical Analyses

Serum creatinine, plasma LDL cholesterol, triglycerides, and HbA_{1c} were measured using standardized methods from venous samples. The urinary albumin excretion rate (UAER) was analyzed by enzyme immunoassay based on three consecutive 24-h urine collections. eGFR was calculated based on serum creatinine using the Chronic Kidney Disease Epidemiology Collaboration equation. An automated validated device was used to measure sitting brachial blood pressure after a 10-min rest.

Baseline DR stage was classified using in-house algorithms on a 0–4 scale based on regular retinopathy screenings at Steno Diabetes Center Copenhagen performed by specifically trained and certified nursing staff under the supervision of ophthalmologists. Five mydriatic nonstereoscopic fundus photos are taken; one macula-centered and four peripheral 45° fundus images. These are combined into a mosaic, grading the macula and periphery separately, according to a modified version of the international classification of DR disease severity scale (10). The presence of microaneurysms, intraretinal hemorrhages, hard or soft exudates, and proliferations are recorded and quantified. Likewise, intraretinal microvascular abnormalities and venous beading are recorded. A weighted quantitation of the distinct retinal pathologies was used to perform staging. Overall stage was defined as the highest stage diagnosed in either eye. Stage 0 is defined as no DR in any eye, stage 1 as mild nonproliferative DR (NPDR), stage 2 as moderate NPDR, stage 3 as proliferative DR (PDR), and stage 4 as PDR with fibrosis. Blind subjects are not screened for retinopathy at Steno Diabetes Center Copenhagen and were therefore excluded. Blindness was defined as visual acuity of less than 1/60, lack of ability to count fingers in front of a white screen at 1 m distance, and lack of ability to see hand motion in front of a white screen at 1 m distance.

Sample Quantification and Identification

The metabolomics analysis is detailed in Tofte et al (11), and the lipidomics analysis is detailed in Tofte et al. (12). For completeness, the analyses are outlined here as follows: Serum samples, stored at −80°C, were analyzed by two different analytical methods. Metabolomics samples were analyzed using a two-dimensional gas chromatography with time-of-flight mass spectrometry. Peak-picking from the raw data was performed with ChromaTOF, and the resulting features were aligned with Guineu (13).

Lipidomics samples were prepared using a modified Folch extraction procedure (14) and analyzed by a previously presented ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry method (15). The raw data were preprocessed with MZmine 2 (16). A complete list of identified metabolites is available in Tofte et al. (11). Finally, the metabolomics and lipidomics data were postprocessed in R software, as described previously (11,12). Lipid species are defined as the number of carbon atoms (indicating total fatty acid chain length) and number double-bonds for the specific species. They are presented as "species (number of carbon atoms:number of double-bonds)."

Within the coverage of the two mass spectrometry platforms, the inclusion of metabolites and lipids in subsequent data analysis was solely based on the certainty of identification and the level of technical precision, thereby not restricting to any particular pathway or prior hypothesis.

Follow-up

Data regarding retinopathy were obtained using local electronic records from Steno Diabetes Center Copenhagen up to 31 December 2016 and were available for 563 subjects. The end points were defined as *1*) progression from any stage to any other stage of DR (any progression); *2*) onset of DR; and *3*) progression from stage 1–2 to stage 3–4 (progression from mild to severe DR). In the case of participants experiencing multiple end points, only the first occurrence was included.

Statistical Analysis

Continuous variables are presented as mean (SD), if normally distributed, and as median (interquartile range [IQR]) if skewed. Before all analyses, skewed variables were $\log_2$ transformed, including all metabolites, to achieve normal distribution. Categorical variables are presented as total number (%). Baseline clinical characteristics were compared across baseline DR status using ANOVA and the χ^2 test for continuous and categorical variables, respectively.

Metabolites and lipid species were analyzed using a narrowing-down approach in relation to DR stages and

outcomes as follows: Cross-sectional relationships between single metabolites or lipid species and baseline DR stages were assessed using multivariate linear regression models adjusted for relevant clinical variables. Thereafter, the single measures were cross-sectionally associated to categories of DR stage and tested using ANCOVA. The Benjamini-Hochberg (BH) method (P_{BH}) (17,18) was used to correct for multiple testing for presented P values throughout the analysis. Metabolites with $P_{BH} < 0.05$ and lipids with $P_{BH} < 0.1$ in the adjusted cross-sectional model were included in survival analysis with the Cox proportional hazards model for end points. All hazard ratios (HRs) are reported per doubling of the metabolite or lipid.

Clinical variables in the adjusted models were age, sex, HbA_{1c}, systolic blood pressure, smoking, BMI, statin treatment, triglycerides, LDL cholesterol, and prescribed antihypertensive medication.

Partial correlation network analysis was done with the R package "huge" (19) using the graphical lasso algorithm (20) coupled with the extended Bayesian information criterion (21). All metabolites were included in inferring the network. Subsequently, the subnetwork of metabolites, which were immediately connected to the four retinopathy-associated metabolites, was visualized with the R package qgraph (22). Edges or the network were colored by the respective partial correlation in the graphical lasso model, and nodes were colored with respective Spearman correlation to the top candidate biomarkers. In both color annotations, red and blue refer to positive and negative correlation, respectively. Size of nodes refers to the respective degree (i.e., the number of associations to other nodes). Statistical analysis and data visualization were performed using R (version 3.4.2).

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

RESULTS

Baseline Characteristics

Baseline characteristics for the participants divided according to baseline DR stages are summarized in Table 1. Most had no DR (n = 141 [22%]) or moderate NPDR (n = 186 [29%]). Compared with no DR, participants with PDR with fibrosis had higher mean (SD) BMI (24.7 [3.5] vs. 26.8 [11.2] kg/m^2), UAER (11.5 [IQR 7.6–22.4] vs. 49.0 [IQR 14.9–231.8] mg/24 h), and systolic blood pressure (127 [(46.1] vs. 135 [20.3] mmHg), and lower eGFR (94.8 [24.7] vs. 64.5 [30.6] mL/min/1.73 m^2). Across groups, age, systolic blood pressure, and UAER were higher with higher DR stage, and the eGFR was lower. Also, the frequency of treatment with antihypertensive drugs and statins was higher with higher DR stage.

Metabolomic Cross-sectional Analyses

A total of 75 metabolite species were identified and passed quality control (Supplementary Table 1). These metabolites were included in the multivariate linear regression models. Four metabolites were positively correlated to baseline DR stage after adjustment for clinical variables and correction for multiple testing: 2,4-DHBA ($P_{BH} < 0.001$), ribonic acid ($P_{BH} = 0.017$), ribitol ($P_{BH} = 0.032$), and 3,4-DHBA ($P_{BH} = 0.036$). Thereafter, all 75 metabolites were included in ANCOVA for baseline DR stages. The same four metabolite levels were significantly increased by higher DR stage after adjustment (ribonic acid, $P_{BH} < 0.001$; 2,4-DHBA, $P_{BH} < 0.001$; ribitol, $P_{BH} = 0.013$; and 3,4-DHBA, $P_{BH} = 0.041$) (Table 2). Figure 1 illustrates the distribution and relative levels of these four metabolites. It is apparent that the levels of all four metabolites increase with higher DR stage. Both 2,4-DHBA and 3,4-DHBA (Fig. 1*B* and *D*) follow the same pattern of lower levels of the metabolite for no DR and NPDR and a nonlinear increase toward higher DR stages and PDR with fibrosis, in particular. In contrast, the levels of ribonic acid and ribitol (Fig. 1*A* and *C*) increase linearly with increasing DR stage.

The partial correlation network of the metabolome connected to the four metabolites is shown in Fig. 2. This network included kidney function–related metabolites (e.g., creatinine and *myo*-inositol), glucose metabolism–related compounds (e.g., citric acid and glycine), fatty acids (e.g., fumaric acid and malic acid), and amino acids (e.g., alanine and serine). Particularly, creatinine and *myo*-inositol, but also glyceryl-glycoside, 4-hydroxybenzeneacetic acid, and fumaric acid, were highly associated with the hub of the four retinopathy-associated metabolites. Except for the amino acids, most of the compounds in the network were positively correlated with the four highlighted metabolites, as indicated by the red color in Fig. 2.

Lipidomic Cross-sectional Analyses

After identification and quality control, 104 lipid species from the following five major lipid classes were included in the analyses: diacyl-phosphatidylcholines (PCs), alkyl-acyl-phosphatidylcholines, lyso-phosphatidylcholines (LPCs), triacylglycerols (TGs), and sphingomyelins. Lipid species are defined as number of carbon atoms (indicating total fatty acid chain length) and number double-bonds for the specific species. They are presented as "species (number of carbon atoms:number of double-bonds)." The investigated lipids are listed in Supplementary Table 2.

TG(50:1) and TG(50:2) were inversely associated with DR grade at baseline after adjustment for clinical covariates, when testing with linear regression analysis ($P_{BH} < 0.05$). Furthermore, LPC(16:1), PC(32:1), TG(14:0/16:0/18:1), TG(50:3), and PC(32:2) were inversely associated with baseline DR grade at a higher false discovery rate of 10%.

Table 1—Baseline characteristics across DR stage

	No DR	Mild NPDR	Moderate NPDR	PDR	PDR with fibrosis	*P*
n (%)	141 (21.8)	90 (14.0)	186 (28.8)	121 (18.8)	107 (16.6)	—
Men, *n* (%)	69 (49.3)	47 (52.2)	120 (64.5)	62 (51.7)	60 (56.1)	0.051
Age, years	46.5 (15.1)	54.9 (11.8)	56.4 (10.7)	59.1 (10.8)	55.8 (10.9)	<0.001
Diabetes duration, years	16.8 (15.5)	32.1 (16.1)	33.1 (12.4)	41.4 (10.8)	41.3 (9.0)	<0.001
Smokers, *n* (%)	29 (20.6)	17 (18.9)	46 (24.7)	20 (16.5)	21 (19.6)	0.495
BMI, kg/m^2	24.7 (3.5)	24.3 (3.7)	25.8 (4.3)	25.8 (3.8)	26.8 (11.2)	0.014
HbA_{1c}, mmol/mol	64.3 (14.7)	61.7 (11.2)	63.9 (11.3)	66.0 (13.8)	65.5 (11.4)	0.130
HbA_{1c}, %	8.0 (1.3)	7.8 (1.0)	8.0 (1.0)	8.2 (1.3)	8.1 (1.1)	0.130
eGFR, mL/min/1.73 m^2	95 (25)	91 (23)	89 (24)	75 (27)	64 (31)	<0.001
UAER, mg/24 h	12 (8–22)	9 (6–24)	15 (8–53)	29 (9–122)	49 (15–232)	<0.001
Systolic blood pressure, mmHg	127 (16)	129 (15)	132 (16)	134 (18)	135 (20)	0.001
Antihypertensive treatment, *n* (%)	58 (41.4)	49 (54.4)	138 (74.2)	109 (90.1)	103 (96.3)	<0.001
Total cholesterol, mmol/L	4.7 (0.8)	4.7 (0.8)	4.7 (0.9)	4.7 (0.9)	4.6 (0.9)	0.673
Triglycerides, mmol/L	0.9 (0.7–1.3)	0.9 (0.7–1.1)	1.0 (0.8–1.4)	0.9 (0.7–1.3)	1.0 (0.7–1.5)	0.055
Statin treatment, *n* (%)	54 (38.6)	44 (48.9)	113 (60.8)	92 (76.0)	81 (75.7)	<0.001

Data are mean (SD) or median (IQR), unless indicated otherwise. *P* values were calculated using ANOVA and the χ^2 test for continuous and categorical variables, respectively.

Table 2—Cross-sectional association between metabolites and lipids and baseline DR grade

Association to DR grade (MLR)	Effect	95% CI	*P*	P_{BH}
Metabolites				
2,4-DHBA	0.097	0.058; 0.135	<0.001	<0.001
Ribonic acid	0.109	0.049; 0.170	<0.001	0.017
Ribitol	0.072	0.028; 0.116	0.001	0.032
3,4-DHBA	0.059	0.022; 0.097	0.002	0.036
Lipids				
TG(50:2)	−0.066	−0.104; −0.028	<0.001	0.042
TG(50:1)	−0.074	−0.118; −0.030	0.001	0.042
PC(32:2)	−0.085	−0.140; −0.029	0.003	0.083
LPC(16:1)	−0.087	−0.146; −0.028	0.004	0.084
TG(14:0/16:0/18:1)	−0.065	−0.109; −0.021	0.004	0.084
TG(50:3)	−0.053	−0.091; −0.016	0.006	0.092
PC(32:1)	−0.080	−0.137; −0.023	0.006	0.092

Association to DR grade (ANCOVA)	*F*	*P*	P_{BH}
Metabolites			
Ribonic acid	7.44	<0.001	<0.001
2,4-DHBA	6.58	<0.001	0.001
Ribitol	4.98	<0.001	0.013
3,4-DHBA	4.18	<0.001	0.041
Lipids			
TG(50:1)	3.97	0.003	0.223
TG(50:2)	3.55	0.007	0.257
TG(49:3)	3.44	0.009	0.257
LPC(16:1)	3.12	0.015	0.354
LPC(16:0)	2.71	0.030	0.544
TG(52:2)	2.62	0.034	0.544
TG(14:0/16:0/18:1)	2.58	0.036	0.544
PC(32:1)	2.44	0.046	0.547
TG(16:0/18:0/18:1)	2.37	0.052	0.547
PC(32:2)	2.29	0.059	0.547

Presented in the top half are multivariate linear regression model effect sizes per increase in DR grade for each metabolite or lipid to baseline DR grade with 95% CI and crude and BH-adjusted *P* values. In the bottom half of the table are ANCOVA *F* values presented for each metabolite and lipid to baseline DR grade with crude and adjusted *P* values. All presented models include the following baseline covariates: age, sex, HbA_{1c}, systolic blood pressure, smoking, BMI, statin treatment, triglycerides, LDL cholesterol, and prescribed antihypertensive medication. MLR, multivariable linear regression.

When investigating with ANOVA, a difference in the lipid level between the DR grades was detected in LPC(16:0) ($P_{BH} < 0.1$). This association was lost after adjustment for the clinical covariates (ANCOVA). In addition to LPC(16:0), medium-sized unsaturated TGs and small LPCs had stronger indicative associations with DR grade than other lipids, as shown in the lipidome-wide heatmap of the ANCOVA *F* statistics (Fig. 3). In particular, LPCs(16:1), as well as TG(49:3), TG(50:1), and TG(50:2), emerged with an indicative association with the DR grade.

Longitudinal Analyses

Metabolites and lipids identified in cross-sectional analyses were thereafter analyzed with compound-specific Cox proportional hazards models for association to any progression, onset of DR, and progression from mild to severe DR. Median follow-up ranged between 5.1 and 5.5 years depending on the end point. The number of events was 133, 47, and 29 for any progression, onset of DR, and progression from mild to severe DR, respectively. For the any progression end point, higher 3,4-DHBA exhibited significance after adjustment for clinical covariates and multiple testing (HR 1.55, 95% CI 1.12–2.15, $P = 0.033$). The other metabolites were not associated with any of the end points neither before nor after adjustment. Although not statistically significant, 2,4-DHBA showed a high HR for progression from mild to severe DR (HR 1.92, 95% CI 0.94–3.93, $P = 0.290$). Unadjusted and adjusted HRs for the metabolites are presented as a forest plot in Fig. 4.

Unlike the metabolites, no lipids were independent risk factors for any of the end points ($P > 0.05$).

DISCUSSION

The current study illustrates an exciting new avenue in characterizing individuals with T1D with DR. In the present cohort we have investigated individuals with long diabetes duration and a broad range of albuminuria, leading to a high proportion of subjects with more severe DR than would be expected in a general clinical population with T1D. We identified four metabolites associated with presence of DR as well as higher 3,4-DHBA as an independent risk marker for progression of DR. Our results were independent of a panel of metabolic risk factors traditionally used for risk stratification of DR in T1D. Therefore, we now argue for the need for further investigation of omics-based risk stratification of DR. Using omics in relation to DR is a relatively new venture, and clinical studies assessing its viability are sparse, especially in subpopulations such as individuals with T1D, and using longitudinal data.

The metabolites identified in this study mainly stem from two etiopathogenic factors, namely, hyperglycemia (ribitol and ribonic acid) and dyslipidemia (2,4- and 3,4-DHBA) (23). Ribitol and ribonic acid are derivatives from ribose, which is highly active in the pentose phosphate pathway and in the production of nucleotides and nucleic acids. Furthermore, sugar alcohols, such as sorbitol, are active in the polyol pathway, which has been identified as a crucial insulin-independent pathway relevant in the onset of DR, and have been suggested as a possible therapeutic target in the treatment of DR (24). The fructose created in this pathway becomes further phosphorylated, resulting in the formation of advanced glycation end products (AGEs), which in turn bind to receptors for AGEs (RAGE)—a known facilitator of DR (25).

DHBAs, on the other hand, which are closely related to the ketone body hydroxybutyric acid, have not been directly associated with any major pathways implicated in the onset of DR or T1D. Other diseases, such as

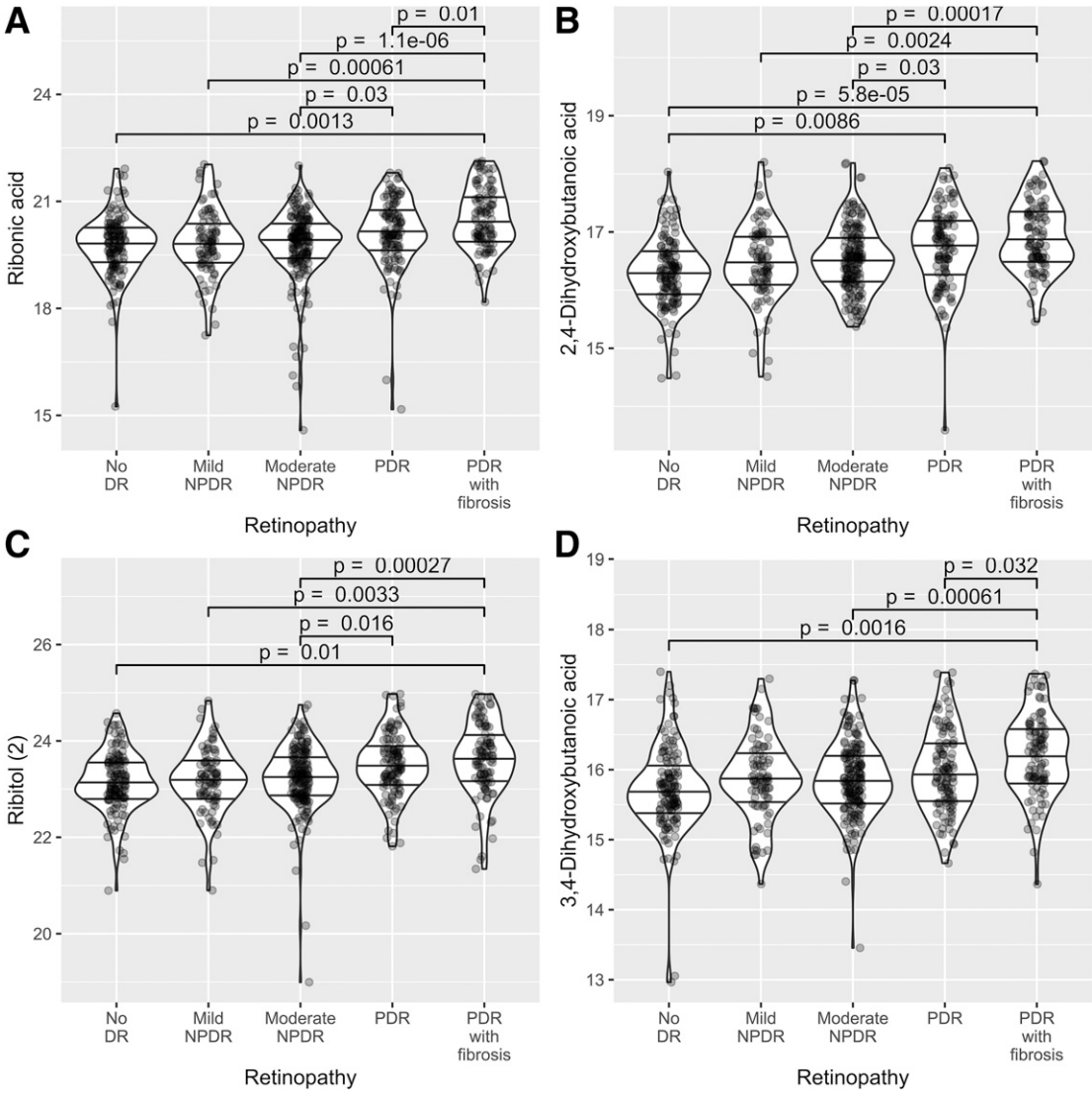

Figure 1—Violin plots show metabolite levels across baseline DR stages for four metabolites—ribonic acid (*A*), 2,4-DHBA (*B*), ribitol (*C*), and 3,4-DHBA (*D*)—where a difference between the stages was detected using ANCOVA. Observations are shown as dots, and their distribution in each of the stages of retinopathy as a violin geom. Pairwise differences between the stages are indicated with *P* values at the top of the figure. The results are from models with adjustment for age, sex, HbA_{1c}, systolic blood pressure, smoking, BMI, statin treatment, triglycerides, LDL cholesterol, and prescribed antihypertensive medication.

succinic semialdehyde dehydrogenase deficiency, an autosomal recessive genetic disease, leads to 4-DHBA aggregation, and, in turn, is associated to severe neurological complications and symptoms (26). In general, ketone bodies are associated with dyslipidemia and high-fat diets (27), which are highly relevant risk factors in the development of diabetic complications. A theory that has drawn recent attention is that DHBAs could be implicated in the butyrate metabolism by the gut microbiota (28), although at present this still calls for more investigation. Similarly, Sumarriva et al. (6) demonstrated that higher plasma carnitine, a metabolite highly present in food that contains meat, was associated to the presence of PDR compared with NPDR. Carnitine is further metabolized by the gut microbiota into trimethylamine-*N*-oxide, which has been associated to cardiovascular and metabolic diseases (29).

Interestingly, visualized in Fig. 2, the subnetwork of metabolites associated with DR shows that all metabolites that correlated significantly with DR in this study all seem strongly associated to *myo*-inositol. Despite *myo*-inositol itself not being associated to DR, in this study, the association to the other metabolites could propose another pathway of DR etiology. *myo*-Inositol is a sugar alcohol, the gastrointestinal absorption and intracellular transport of which has been shown to be impaired in individuals with diabetes (during hyperglycemia) (33). Furthermore, while previously thought mainly to be expressed in renal tissue, recent studies show evidence of *myo*-inositol activity in extrarenal tissue, such as retinal and lens epithelium as

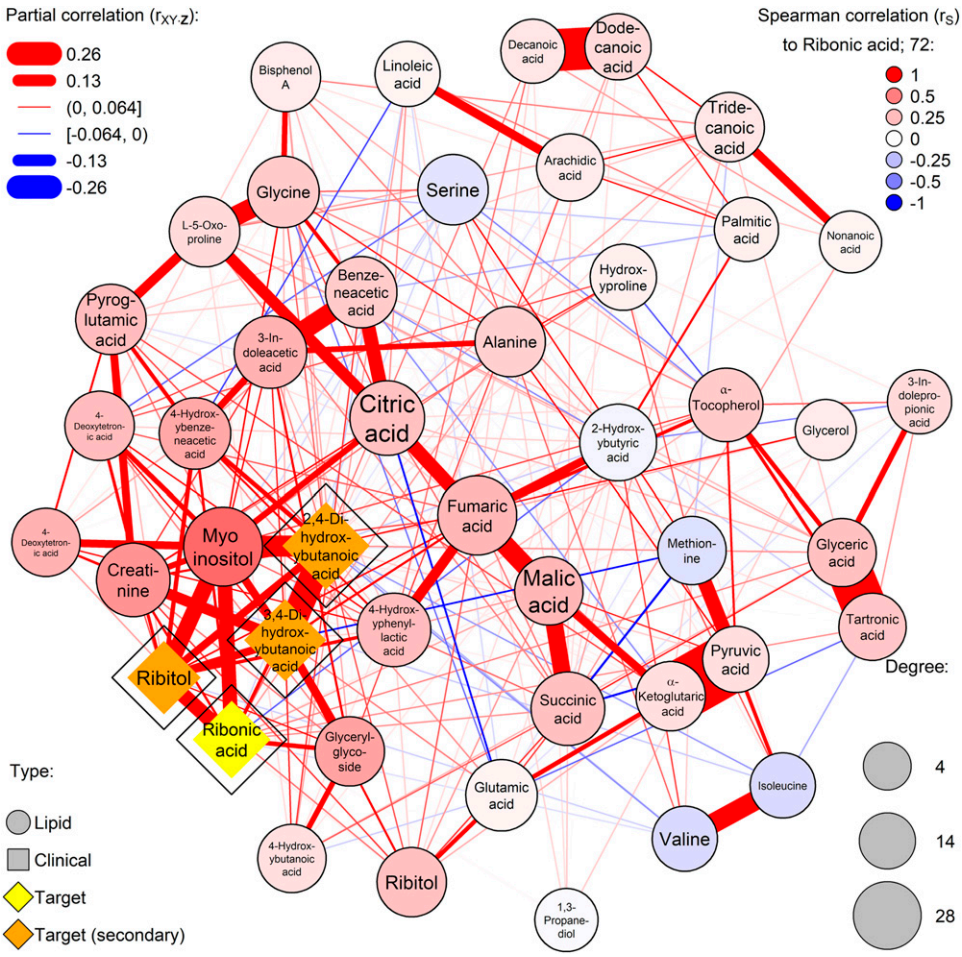

Figure 2—Partial correlation network shows associations in the measured metabolome. Metabolites with cross-sectional association to retinopathy (ribitol, ribonic acid, 2,4-DHBA and 3,4-DHBA) are indicated as diamond-shaped nodes. The node color of the other metabolites (circular nodes) indicates the Spearman correlation to ribonic acid. Partial correlations (i.e., independent associations) are indicated by lines between the metabolites, where thickness and color of the line, respectively, indicate the strength and the sign of the association (red: positive, blue: inverse). Additionally, the size of the node indicates the degree of the node (i.e., the number of associations with the metabolite).

well (34,35), and has additionally been associated to existing DR in individuals with type 2 diabetes (36).

Our metabolomic results stand well in relation to a study by Chen et al. (5), describing similar metabolites associated to DR in a cross-sectional study including individuals with type 2 diabetes with DR ($n = 40$) or without DR ($n = 40$) in the Singapore Indian Eye Study. They demonstrated that 3,4-DHBA as well as ribose were significantly higher in the DR group compared with the group without DR. Furthermore, similar results were found for 2-deoxyribonic acid as well as for several other sugars and sugar derivates (5). As such, we can partly validate these results in our larger population of individuals with T1D, and additionally, we have shown that 3,4-DHBA was a risk marker for progression of DR during follow-up. The inherent issue with omics discovery studies being explorative is that replication of results is necessary across populations and cohorts, but is often difficult, especially due to platform heterogeneity. The study by Chen et al. (5) strengthens the findings in our study suggesting that 3,4-DHBA and ribose derivatives are valid risk markers of DR. However, Lin et al. (8) showed that branched-chain, aromatic, and glucogenic amino acids, such as leucine, valine, tyrosine, and alanine, were positively associated with diabetic microangiopathy in type 2 diabetes. These amino acids were also investigated in our study; however, we could not confirm the results, possibly due to the heterogeneity between type 1 and type 2 diabetes.

In the case of lipids, some, such as LDL cholesterol and TGs, have throughout many years been comprehensively studied and have proved to be robust risk markers for vascular disease in T1D. In addition, studies targeting dyslipidemia with fibrates have found beneficial effects on retinopathy (37,38). Therefore, the concept of applying lipidomic strategies into finding novel markers to strengthen the identification and prediction of vascular risk is not implausible. In our panel of lipids, we were only able to

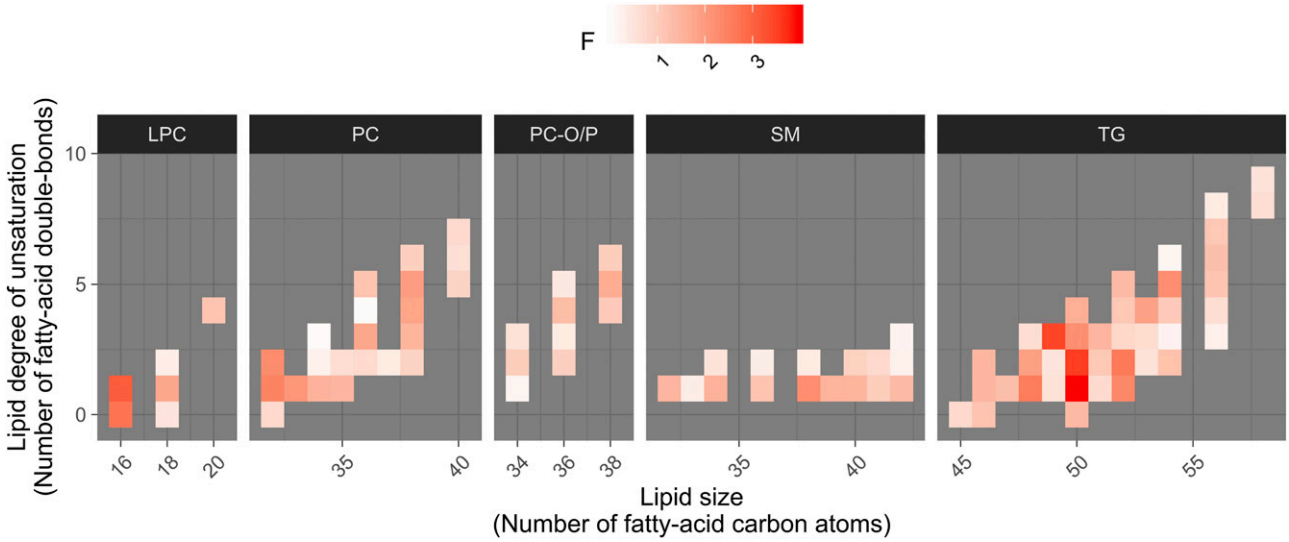

Figure 3— Heatmaps of the ANCOVA model *F* statistics across the entire lipidomic panel in relation to DR stage. The results are from models with adjustment for age, sex, HbA_{1c}, systolic blood pressure, smoking, BMI, statin treatment, triglycerides, LDL cholesterol, and prescribed antihypertensive medication. Lipid species are grouped according to the lipid classes in each panel. Each cell represents one lipid species. On the *y*-axis is number of double-bonds for the specific species (indicating level of saturation), and on the *x*-axis is the number of carbon atoms (indicating total fatty acid chain length). PC-O/P, alkyl-acyl-phosphatidylcholines; SM, sphingomyelins.

identify three TGs negatively correlated with DR stage in our linear models using a 5% α-level, adjusted for, among others, baseline LDL cholesterol, triglycerides, and BMI. However, these associations could not be replicated in ANCOVA models, arguably in part due to nonlinear trends for different lipids across DR stages.

Comparing the present results in relation to other studies is difficult because very few have investigated DR.

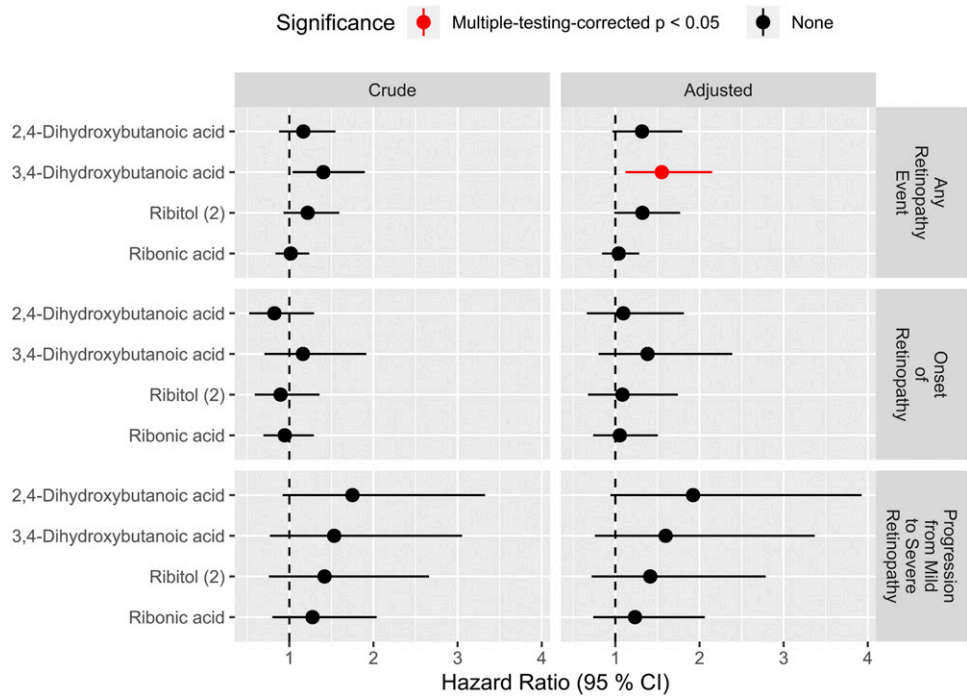

Figure 4— Forest plot of multiple testing–corrected HRs of the selected metabolites for three retinopathy events: any retinopathy, onset of retinopathy, and progression from mild to severe retinopathy (top, middle, and bottom panels, respectively). Adjusted model (right) is adjusted for age, sex, HbA_{1c}, systolic blood pressure, smoking, BMI, statin treatment, triglycerides, LDL cholesterol, and prescribed antihypertensive medication. The crude model (left) is without these adjustments.

One study found stearic acid, *trans* oleic acid, linoleic acid, arachidonic acid, and free cholesterol in circulation to be significant differentiators between preclinical DR, NPDR, and PDR (39). Likewise, Schwartzman et al. (40) identified several free-fatty acid autacoids in vitreous humor as markers of PDR in T1D.

PDR is demonstrated to be associated to an impaired blood-brain barrier (BBB), and early damage to the BBB is hypothesized to be a predictor of progression to more advanced stages of DR (41). However, the mechanisms for the association surrounding BBB impairment and DR are not understood, and the association to circulating biomarkers has not been described. Hogan et al. (42) demonstrated that various polyunsaturated fatty acids and sphingolipids were associated with impaired BBB in traumatic brain injury in rats, but our results do not support the association between these and DR.

Moving outside of purely metabolomic and lipidomic studies, a substantial amount of research has been performed on the proteome and its effect on DR risk and risk progression, however, primarily in small samples with largely nonreplicated results (43).

This study is not without limitations. The DR staging during follow-up did not take laser surgery or vascular endothelial growth factor injections into account, because it was based only on changes in DR stage from baseline. Furthermore, no data on concomitant medication throughout the follow-up were available, and as such, there are no data on how treatment with statins, antihypertensive medication, or insulin has changed during follow-up. In addition, no information on lifestyle parameters, which could have influence on lipid composition, was available at baseline. Finally, the lack of a validation population is another limiting factor. Nonetheless, the sizable strengths of this study are, firstly, the large, well-defined cohort of individuals with T1D, including 7 years of longitudinal data, and secondly, a comprehensive metabolomic and lipidomic analysis regarding presence of and changes in DR in T1D.

In summary, we identified four metabolites and three lipids with an association to the DR stage: ribonic acid, ribitol, and two DHBAs were associated with DR stage, and three triglycerides were negatively correlated with the DR stage. Furthermore, we have identified 3,4-DHBA as an independent risk marker for progression in DR stage. Our results may serve as a basis for further studies regarding sugar metabolism, hydroxy acids, and lipids in relation to diabetic complications such as retinopathy, because more investigative studies are needed before these markers can be clinically applied.

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Author Contributions. V.R.C. wrote the manuscript and is responsible for the integrity of the work as a whole. V.R.C., T.S., I.M., L.A., S.T., T.W.H., C.L.-Q., and P.R. conceived and designed the research. V.R.C., T.S., I.M., L.A., S.T., T.W.H., C.L.-Q., and P.R. analyzed and interpreted the data. V.R.C., T.S., I.M., L.A., S.T., T.W.H., C.L.-Q., and P.R. critically revised the manuscript for key intellectual content. T.S. performed the statistical analysis. P.R. obtained funding and supervised the study. All authors approved the final version of the manuscript. V.R.C. 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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