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Plasma Metabonomic Profiling of Diabetic Retinopathy

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Diabetic retinopathy (DR) is the most common microvascular complication of diabetes and the leading cause of visual impairment in working-age adults. Patients with diabetes often develop DR despite appropriate control of systemic risk factors, suggesting the involvement of other pathogenic factors. We hypothesize that the plasma metabolic signature of DR is distinct and resolvable from that of diabetes alone. A nested population-based case-control metabonomic study was first performed on 40 DR cases and 40 control subjects with diabetes using gas chromatography–mass spectrometry. Eleven metabolites were found to be correlated with DR, and the majority were robust when adjusted for metabolic risk factors and confounding kidney disease. The metabolite markers 2-deoxyribonic acid; 3,4-dihydroxybutyric acid; erythritol; gluconic acid; and ribose were validated in an independent sample set with 40 DR cases, 40 control subjects with diabetes, and 40 individuals without diabetes. DR cases and control subjects with diabetes were matched by HbA_{1c} in the validation set. Activation of the pentose phosphate pathway was identified from the list of DR metabolite markers. The identification of novel metabolite markers for DR provides insights into potential new pathogenic pathways for this microvascular complication and holds translational value in DR risk stratification and the development of new therapeutic measures.

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes and the leading cause of visual impairment in working-age adults worldwide (1,2). The global prevalence of diabetes is rising and the number of people with diabetes is projected to increase by 54% in 2030, compared with 2010 (3). The public health burden of diabetes and DR would thus increase correspondingly. The major risk factors of DR are poor glycemic control and hypertension, as well as the duration of diabetes (4,5), but their relative importance varies between studies (2,6). Although the risks of DR progression and vision loss are reduced with intensive control of risk factors (7,8), many patients with diabetes continue to develop complications despite tight glycemic and blood pressure control. There is increasing evidence to suggest that "metabolic memory" is responsible for this observation. The term metabolic memory refers to the persistent epigenetic modifications caused by early exposure to hyperglycemia that, in turn, predispose individuals to the development of diabetes complications even after good glycemic control

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is achieved (9). More recently, Zavrelova et al. (10) found that the progression of DR in a treated cohort occurs in distinct patterns, ranging from a stable condition that is free from DR to rapid progression to the proliferative DR within 6 years. The mechanisms responsible for these patterns in disease progression are unclear (11). The discovery of biomarkers that characterize the different developmental phenotypes of DR thus become important as these biomarkers may provide insight on the pathogenic pathways that are currently unknown and may allow for the clinical stratification of patients for DR monitoring and treatment (5).

The variation in the metabolome represents the interplay of genetic and environmental factors and provides information that is complementary to genomic, transcriptomic, or proteomic data. The study of metabolic phenotypes (metabotypes) in association with disease states may reveal new knowledge in disease mechanism and pathophysiology (12). Recent metabonomic studies have uncovered plasma and sera metabolic signatures associated with, or predictive of, impaired glucose tolerance and diabetes (13–22). Furthermore, DR is a complex disease where findings from genome-wide association studies have not been conclusive (23). We postulate that a distinct metabolic signature of DR exists and can be resolved from that of diabetes alone. Barba et al. (24) identified metabolite markers of DR in the vitreous humor. However, the invasive nature of vitreous sampling limits study replication and the translational potential of any biomarkers identified from vitreous fluid. In contrast, plasma or sera have remained the biofluid of choice in metabonomic studies. With these biological matrices, several research groups have reported metabolite markers of global (25,26) and targeted (27–29) metabonomic analysis. These studies, however, did not account for confounders and comorbidities, such as medication use and kidney disease. The current study's objective is to investigate if the plasma metabotype of DR is resolvable from the metabolic perturbations associated with underlying diabetes and to identify novel metabolite markers of DR.

RESEARCH DESIGN AND METHODS

Sample Selection

We first conducted a nested population-based case-control study on 40 patients with diabetes with DR and 40 patients with diabetes without DR (discovery set) selected from banked plasma collected as part of the Singapore Indian Eye Study (SINDI) (30). Metabolite markers of DR identified from the discovery set were separately quantified in a separate sample set with 40 patients with diabetes and DR, 40 patients with diabetes and without DR, and 40 participants without diabetes (validation set).

For the discovery set, cases were 40 participants with type 2 diabetes with moderate nonproliferative DR (level 43 on the Early Treatment of Diabetic Retinopathy Study [ETDRS] scale) or worse in at least one eye, documented from retinal photographs (31). Equal numbers of samples were selected from participants who had been diagnosed with diabetes for 10 years or less and for over 10 years. Control subjects were 40 participants with diabetes who had no DR, matched to cases by diabetes duration (in 5-year bands).

For the validation set, participants with chronic kidney disease were excluded from selection. Cases were 40 participants with type 2 diabetes with moderate nonproliferative DR (level 43 on the ETDRS scale) or worse in at least one eye. Control subjects were 40 participants with diabetes who had no DR, matched to cases by HbA_{1c} levels (in 0.5% bands), age (in 5-year bands), and sex. A total of 40 participants with no diabetes were also selected for the validation set, matched to DR cases by age and sex.

SINDI Protocol

SINDI was a population-based study of Indian adults aged 40 years and older living in Singapore. The study was approved by the SingHealth Institutional Review Board and conducted in accordance to the Declaration of Helsinki, and written informed consent was obtained from all participants. Detailed population selection and methodology have been published previously (30). Briefly, from 2007 to 2009, the study was conducted in the southwestern part of Singapore using an age-stratified random sampling strategy. Of these, 4,497 individuals were deemed eligible to participate and 3,400 participants took part in the study, giving a 75.6% participation rate (31).

Participants had a comprehensive examination with a standardized questionnaire and systemic and ocular examination and had plasma and serum samples collected and stored. Diabetes was defined as self-report of a previous diagnosis of the disease by a doctor, use of diabetes medication, or HbA_{1c} of 6.5% (48 mmol/mol) or greater (32). DR was graded from retinal photographs using a scale modified from the Airlie House classification system. Clinical and biochemical variables such as BMI, total cholesterol, LDL cholesterol, HDL cholesterol, HbA_{1c}, and blood pressure (BP) were measured from standardized protocols. Chronic kidney disease was defined as having an estimated glomerular filtration rate (eGFR) of less than 60 mL/min/1.73 m^2.

Plasma Collection Protocol

K_2EDTA tubes were used to collect blood samples. Tubes were kept on ice and transported to the laboratory within the same working day. Tubes were centrifuged at 1,000*g* for 10 min (4°C) to separate plasma from whole blood. Plasma aliquots were stored at −80°C.

Gas Chromatography–Mass Spectrometry Metabonomic Profiling

Plasma samples (100 μL aliquots in microcentrifuge tubes) were thawed to room temperature (25°C). D-27 myristic acid (20 μL of 200 μg/mL solution in methanol) and 300 μL of methanol were added to each sample. Samples were mixed for 15 min at room temperature and centrifuged for 10 min at 16,000*g* (4°C). The top 300 μL of each supernatant was transferred to a glass tube and dried under nitrogen gas. The dried samples were resuspended in 100 μL of toluene, vortexed vigorously for 10 s, and dried

again under nitrogen. A two-step derivatization method was used for chemical derivatization of the metabolites. Samples were first incubated with 50 μL of 2% methoxyamine chloride in pyridine (Pierce Biotechnology) for 1.5 h at 60°C. Next, 50 μL of N-methyl-N-trifluoroacetamide (Thermo Scientific) was added and the samples were incubated again for 1 h at 60°C. A total of 80 μL of derivatized samples were transferred to silanized glass vials for gas chromatography–mass spectrometry (GC-MS) analysis. Ten aliquots of pooled K_2EDTA human plasma (batch 052511; Innovative Research, Inc.), three aliquots of distilled water, and 10 mmol/L glucose in distilled water were also similarly prepared. Two pooled plasma samples were injected before the first study sample and one pooled plasma sample was injected after every 10 study samples to act as quality controls (QC). The blanks and glucose standards were injected at the beginning, at the middle, and at the end of the sample batch.

GC-MS analysis was performed using an Agilent 6890N gas chromatograph (Agilent Technologies) coupled to a LECO Pegasus III (4D) GC × GC/MS time-of-flight mass analyzer operating in GC-MS mode (LECO Corp.). Sample injection was performed by a CTC PAL autosampler (CTC Analytics AG). The injection temperature was 250°C and injection volume was set to 1 μL, with a spilt ratio of 1:2. A capillary column with DB-1 stationary phase of 21.5 m × 0.25 mm, 0.25-μm film thickness (Agilent Technologies) was used with a constant helium carrier gas flow of 1.5 mL/min. The temperature gradient was held at 70°C for 2 min, increased at a rate of 10°C/min to 310°C, and held at 310°C for 6 min. The transfer line and ion source were set at 280°C and 250°C, respectively. Mass spectra were acquired after a delay of 240 s postinjection, from mass/charge ratio of 45 to 600 at 20 Hz using ionization energy of 70 eV and a detector voltage of 1,650 V.

Data Processing

Deconvolution and peak finding were performed with ChromaTOF (version 4.44, LECO Corp.) using *1*) a signal-to-noise threshold of 150, *2*) a minimum peak width of 2.5 s, and *3*) a minimum of two apexing masses. Instrument performance was assessed using the peak area of D-27 myristic acid, the internal standard.

Peak alignment was performed with the "calibration" function in ChromaTOF (33). A reference table was first built using data obtained from plasma samples of participants who had declared no medication usage. There were three such participants among the selected samples. Samples from an additional six participants with diabetes who had declared no medication usage were analyzed for the purpose of building the reference table. Each peak was only included in the reference table if *1*) it was detected in at least seven out of nine medication-free samples (78%) and *2*) its average integrated peak area was at least five times greater than that of the glucose standards and blanks. This ensured that the peaks in the reference table included endogenous metabolites as represented in the medication-free samples and excluded xenobiotics such as drugs and their associated metabolites. Peaks derived from glucose were saturated in most samples and were thus excluded from the reference table. Furthermore, plasma glucose levels in the DR group were not significantly different from control subjects with diabetes in this study.

Peak data from DR patients and control subjects with diabetes were then aligned to the reference table using a retention time tolerance of 3 s. Retention times were converted to Kovats retention indices using C8-C40 alkane standards (Sigma-Aldrich). Peaks with missing values in more than 20% of the samples were discarded. For peaks with missing values in less than 20% of the samples, the missing values were filled with half of the lowest detected peak area. The raw peak area was normalized to the sum of peak areas within the 90th percentile of peak area distribution in each sample, using the median sample as the denominator in the calculation of normalization factors. Normalized data were then log transformed (base 2). Peaks with coefficient of variation (CV) greater than 30% in the QC samples were not considered for further analysis.

Statistical Analysis

Principal component analysis (PCA) with unit variance scaling was performed to evaluate the presence of dominating trends in the peak data. Mann-Whitney *U* tests were first performed to compare the DR group with control subjects with diabetes. To correct for multiple testing, false discovery rates (FDR) was calculated using *q* values (34). Metabolites were considered significant if their *q* values were ≤ 0.2, and if their fold changes were >1.2 or <0.8. The area under the curve (AUC) in receiver operating characteristic (ROC) analysis was calculated to evaluate the discriminating power of the metabolite markers (35). Logistic regression models were fit to evaluate the association of metabolite peak areas with the presence of DR. Odds ratios with 95% CI were calculated based on 1 SD change in metabolite peak areas. Clinical variables representing known risk factors of DR (4,5), such as HbA_{1c}, were added as covariates to logistic regression models to calculate adjusted odds ratios. Adjusted odds ratios were also calculated for dominating trends that were identified from PCA and for major classes of diabetes medications. PCA was performed using SIMCA (Umetrics AB), all other statistical tests were performed in the R environment.

Metabolite Identification

Experimentally obtained spectra were searched against entries in the National Institute of Standards and Technology (NIST) library using ChromaTOF. Metabolite identities were confirmed if both the forward and reverse similarity scores were above 800 and the Kovats retention index was within 2% from the database entries. Peaks that could not be identified in the NIST library were exported to text spectra and searched against the Golm Metabolome Database (36). Ambiguous identifications were resolved by injection of derivatized analytical standards. For metabolites forming multiple derivatization peaks, the peaks with lower

CV in QC samples were reported. Peaks with unresolvable identifications were reported as mixtures of compounds.

Pathway Analysis

Pathway analysis was performed with MetaboAnalyst (37). The list of identified metabolites with $P < 0.05$ and a fold change >1.2 or <0.8 were used as input. Canonical pathways were considered to be significantly enriched in the data set if their FDRs were <0.2.

Metabolite Quantitation in Validation Set

Standards for 1,5-gluconolactone; erythritol; gluconic acid; lactose; maltose; ribose; and trehalose were purchased from Sigma-Aldrich. The compounds 2-deoxyribonic acid and 3,4-dihydroxybutyric acid were synthesized in-house and characterized by proton nuclear magnetic resonance. Synthesis methods are described in the Supplementary Data. Calibration curves were constructed with standard solutions from 0.050 to 2.00 μg/mL. Plasma samples were similarly derivatized and analyzed by GC-MS. Besides the column length (28.5 m) and detector voltage (1,800 V), all other settings were the same as those used for the discovery metabonomic profiling. Kruskal-Wallis tests (nonparametric ANOVA) were performed to identify differences in plasma metabolite concentrations between groups.

RESULTS

Sample Characteristics

The clinical characteristics of participants selected for discovery metabonomic profiling are shown in Table 1. Sample groups were comparable for most metabolic and clinical characteristics, such as BMI, total cholesterol, LDL cholesterol, and HDL cholesterol. Participants with DR had higher HbA_{1c} than the control subjects with diabetes, mirroring typical epidemiological findings (4,31). Although hypertension identified as a risk factor for DR in the SINDI cohort (4,5,31), the increased systolic BP between DR cases and control subjects with diabetes in our study was not statistically significant.

Data Table and Chemometric Analysis

There were 263 peaks in the reference table constructed from medication-free samples and 258 were present in at least 80% of the samples. The CV of D-27 myristic acid, the internal standard, was 7.9%, and 193 peaks had a CV of less than 30% in the QC samples.

The PCA model constructed from aligned peak data was optimized at 5 PCs, with R^2 and Q^2 values of 0.48 and 0.27, respectively. The first and second PCs explain 15% and 6% of model variation, respectively. Most samples from participants with chronic kidney disease are located toward the positive scores along the first PC (Supplementary Fig. 1*A*). When labeled by HbA_{1c} values, the majority of samples with greater HbA_{1c} levels are found on the positive axis of the second PC (Supplementary Fig. 1*B*). These clustering trends show that chronic kidney disease and glycemic control are significant contributors to the overall plasma metabotype of people with diabetes and could be confounders in metabonomic analysis.

Table 1—Clinical characteristics of samples selected for discovery metabonomic profiling

Clinical characteristics	DR cases	Control subjects with diabetes	*P* value
N	40	40	—
Sex (male)	20	23	0.501†
Age (years)	59 (53–66)	62 (52–69)	0.846‡
Diastolic BP (mmHg)	74 (68–81)	77 (68–83)	0.516‡
Systolic BP (mmHg)	146 (132–155)	133 (122–146)	0.086‡
BMI (kg/m^2)	24.5 (23.0–28.1)	28.6 (23.4–30.3)	0.070‡
Serum creatinine (μmol/L)	84 (71–134)	80 (58–117)	0.092‡
Blood glucose, random (mmol/L)	10.4 (6.8–14.6)	8.3 (6.9–11.2)	0.109‡
Total cholesterol (mmol/L)	4.50 (3.99–5.39)	4.38 (3.66– 5.25)	0.320‡
LDL cholesterol (mmol/L)	2.84 (2.17–3.49)	2.59 (2.08–3.27)	0.332‡
HDL cholesterol (mmol/L)	0.98 (0.78–1.23)	1.00 (0.84–1.23)	0.486‡
Triglycerides (mmol/L)	1.79 (1.39–2.59)	1.35 (0.94–2.19)	0.076‡
HbA_{1c} (%)	8.2 (7.4–10.2)	7.4 (6.5–8.0)	0.001‡
Duration of diabetes (years)	12 (7–21)	11 (5–19)	0.374‡
Urine ACR (μg/mg creatinine)	46.0 (16.8–161.6)	25.7 (13.2–56.4)	0.053‡
eGFR (mL/min/1.73 m^2)	68.7 (44.6–95.9)	87.5 (50.7–100.9)	0.141‡
Microalbuminuria (*n*)	21	14	0.115†
Macroalbuminuria (*n*)	5	2	0.235†
Chronic kidney disease (*n*)§	16	11	0.237†
On diabetes medication (*n*)	34	32	0.556†

Data are median (interquartile range), unless stated otherwise. †Pearson χ^2 test. ‡Mann-Whitney *U* test. §Defined as eGFR below 60 mL/min/1.73 m^2.

Metabolite Markers of DR

There were 14 candidate metabolites identified from discovery metabonomic profiling ($P < 0.05$, Mann-Whitney U test), and 11 met a q value cutoff of 0.2 after correction for multiple testing (Table 2). DR samples had decreased levels of 1,5-anhydroglucitol and increased levels of 1,5-gluconolactone; 2-deoxyribonic acid; 3,4-dihydroxybutyric acid; erythritol; gluconic acid; lactose/cellobiose; maltose/trehalose; mannose; ribose; and urea. A heatmap showing the relative peak area distribution of these metabolites in DR cases and control subjects with diabetes is shown in Fig. 1. Analytical information of all identified metabolite markers are provided in Supplementary Table 1.

The odds ratios of the metabolite markers in the basic logistic regression models and those adjusted for systolic BP, HbA_{1c} levels, and diabetes duration are shown in Table 3. Although HbA_{1c} was a significant covariate for all metabolite markers ($P < 0.05$ in likelihood ratio test), adjusted odds ratios for 1,5-anhydroglucitol; 1,5-gluconolactone; 2-deoxyribonic acid; gluconic acid; lactose/cellobiose; and urea remained significant. Increased systolic BP and diabetes duration are known risk factors of DR (4,5) but these factors were not significant covariates for any metabolite markers in our study. The metabolite markers 1,5-gluconolactone; 2-deoxyribonic acid; gluconic acid; lactose/cellobiose; and urea retained significant odds ratios when adjusted for systolic BP, HbA_{1c} levels, and diabetes duration together (Table 3). Adjusted odds ratios for age, sex, BMI, diastolic BP, serum creatinine, glucose, triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol, urine albumin-to-creatinine ratio (ACR), and eGFR are shown in Supplementary Tables 2–4. Age, sex, triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol were not significant covariates for any metabolite marker (Supplementary Tables 2–4).

The number of participants with prescriptions for each class of diabetes medication is shown in Supplementary Table 5. Biguanides, sulfonylureas, thiazolidinediones, and α-glucosidase inhibitors were not significant covariates for any metabolite marker, and insulin reduced the significance of only erythritol (Supplementary Table 6).

Validation of Metabolite Markers

The reproducibility of metabolite markers identified from discovery metabonomic profiling was investigated in another set of samples selected from the SINDI cohort. To minimize the contributions of potentially confounding kidney disease and glycemic control, participants with eGFR below 60 mL/min/1.73 m^2 were excluded, and control subjects with diabetes were matched to DR cases for HbA_{1c}. Samples from individuals without diabetes were also analyzed to establish reference ranges of these metabolites. The clinical characteristics of participants selected for the validation set are summarized in Supplementary Table 7.

In the validation set, DR cases showed increased plasma concentrations of 2-deoxyribonic acid; 3,4-dihydroxybutyric acid; erythritol; gluconic acid; and ribose when compared with control subjects with diabetes (Table 4). In particular, control subjects with diabetes also showed increased plasma concentrations of gluconic acid when compared with individuals without diabetes (Fig. 2). The previously unresolved metabolite pairs, lactose/cellobiose and maltose/trehalose were separated with the longer analytical column. Cellobiose was not detected in plasma samples. Trehalose was detected only in 26 DR cases, 22 control subjects with diabetes, and 4 individuals without diabetes and was excluded from further statistical analysis. Mannose was not quantified as it could not be resolved from glucose in this set of analyses.

DISCUSSION

Metabolite Markers Complementary to Known Risk Factors

It is relevant to identify metabolite markers that are complementary to known risk factors, such as glycemic control (4,5), to improve existing risk stratification in DR-free patients with diabetes and those with the early stages of DR. A panel

Table 2—Metabolite markers identified from discovery metabonomic profiling

Metabolites	Fold change*	*P* value	*Q* value	AUC†
1,5-Anhydroglucitol‡	0.50	<0.001	0.03	0.74 (0.63–0.84)
1,5-Gluconolactone	1.45	0.001	0.05	0.71 (0.60–0.83)
2-Deoxyribonic acid	1.60	0.007	0.12	0.68 (0.56–0.79)
3,4-Dihydroxybutyric acid	1.26	0.019	0.20	0.65 (0.53–0.78)
Erythritol	1.25	0.019	0.20	0.63 (0.51–0.76)
Gluconic acid	1.57	0.001	0.03	0.72 (0.61–0.84)
Lactose/cellobiose	1.75	0.010	0.13	0.67 (0.55–0.79)
Maltose/trehalose	1.74	0.003	0.08	0.70 (0.58–0.81)
Mannose	1.21	0.009	0.13	0.67 (0.55–0.79)
Ribose	1.42	0.016	0.19	0.66 (0.54–0.78)
Urea	1.35	0.004	0.08	0.69 (0.57–0.80)

*Values above 1 indicate higher levels in DR group. †AUC in ROC analysis (95% CI). ‡Classification in ROC analysis reversed for metabolites with fold change below 1.

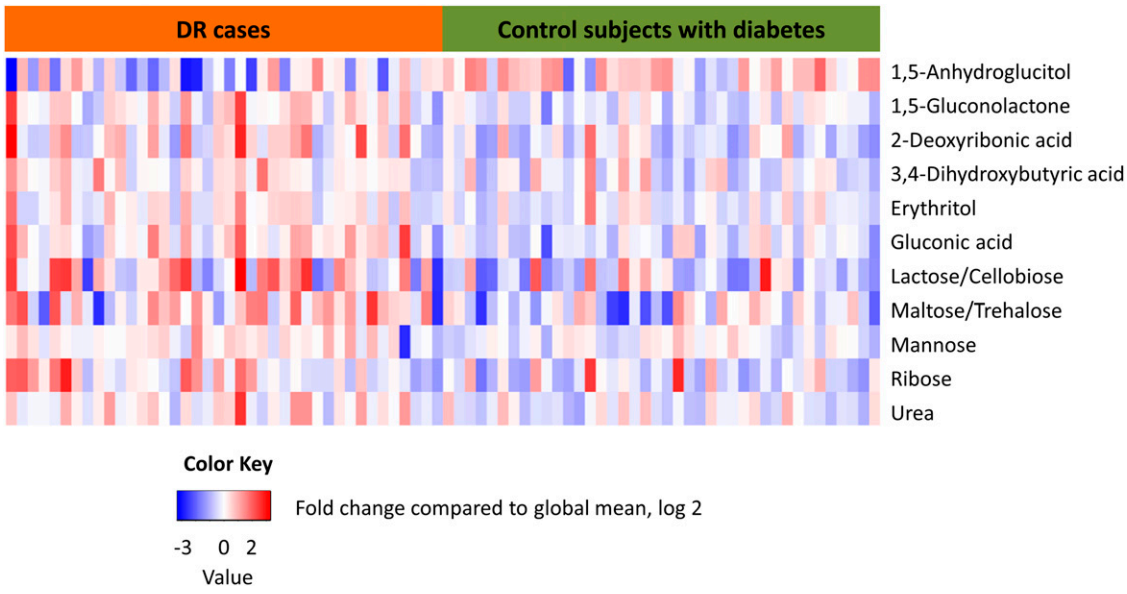

Figure 1—Heatmap showing relative peak areas of metabolite markers identified from discovery metabonomic profiling.

of 11 metabolite markers was identified from discovery metabonomic profiling (Fig. 1 and Table 2). Several of these metabolite markers not only are independent of HbA_{1c} but also remain statistically significant when adjusted for known metabolic risk factors for DR and potentially confounding kidney disease. We found that 1,5-gluconolactone; 2-deoxyribonic acid; gluconic acid; and urea retain significant odds ratios when adjusted for key clinical variables, such systolic BP, HbA_{1c}, duration of diabetes, urine ACR, and eGFR (Table 3 and Supplementary Table 4).

Metabolite Markers of DR Are Distinct From Diabetes

Increased levels of branched-chain (18–20) and aromatic (16–19) amino acids in plasma and sera have been identified as markers and predictors of type 2 diabetes, whereas decreased levels of glycine were found to be correlated with diabetes (19,21). Our results from discovery metabonomic profiling showed no significant differences in the levels of these amino acids between DR cases and control subjects with diabetes (Supplementary Table 8). This contrast is likely due to the definition and choice of the control group. In the aforementioned studies, control samples were derived from healthy people, whereas the control subjects in the discovery set were sampled from people with diabetes who have potentially had pre-existing increased catabolism of branched-chain and aromatic amino acids. In addition, the majority of the participants in our study had been prescribed with diabetes-controlling

Table 3—Odds ratios (95% CI) of metabolite markers adjusted for systolic BP, HbA_{1c}, and diabetes duration

		Odds ratios (95% CI) in adjusted models with covariates			
Metabolites	Odds ratios (95% CI) in basic model	+ Systolic BP	+ HbA_{1c}	+ Diabetes duration	+ Systolic BP, HbA_{1c}, and diabetes duration
1,5-Anhydroglucitol	0.37 (0.19–0.65)*	0.36 (0.19–0.64)*	0.51 (0.25–0.97)*‡	0.37 (0.19–0.64)*	0.54 (0.26–1.07)†
1,5-Gluconolactone	2.40 (1.38–4.65)*	2.58 (1.46–5.05)*	2.28 (1.24–4.78)*‡	2.38 (1.36–4.65)*	2.19 (1.17–4.63)*‡
2-Deoxyribonic acid	2.08 (1.25–3.76)*	2.22 (1.32–4.11)*	2.14 (1.22–4.14)*‡	2.10 (1.24–3.86)*	2.00 (1.13–3.92)*‡
3,4-Dihydroxybutyric acid	1.61 (1.01–2.70)†	1.67 (1.05–2.80)*	1.51 (0.91–2.59)‡	1.59 (0.99–2.67)†	1.49 (0.89–2.57)‡
Erythritol	1.68 (1.04–2.85)*	1.75 (1.08–2.97)*	1.71 (1.02–3.05)†‡	1.67 (1.02–2.87)*	1.59 (0.93–2.85)†‡
Gluconic acid	2.65 (1.50–5.33)*	2.76 (1.55–5.57)*	2.04 (1.10–4.20)*‡	2.64 (1.49–5.31)*	1.96 (1.06–4.03)*‡
Lactose/cellobiose	1.78 (1.10–3.01)*	1.93 (1.19–3.29)*	1.92 (1.13–3.51)*‡	1.77 (1.09–3.02)*	1.92 (1.10–3.59)*‡
Maltose/trehalose	2.14 (1.29–3.84)*	2.01 (1.21–3.57)*	1.46 (0.80–2.82)‡	2.17 (1.30–3.91)*	1.34 (0.70–2.62)‡
Mannose	1.81 (1.09–3.27)*	1.65 (1.00–2.93)†	1.34 (0.74–2.35)‡	1.86 (1.11–3.38)*	1.29 (0.73–2.25)‡
Ribose	1.65 (1.02–2.80)*	1.68 (1.04–2.85)*	1.49 (0.89–2.64)‡	1.63 (1.01–2.78)†	1.43 (0.84–2.58)‡
Urea	1.95 (1.18–3.48)*	2.17 (1.30–3.95)*§	2.50 (1.39–4.98)*‡	1.94 (1.17–3.47)*	2.44 (1.34–4.93)*‡

*P (model) < 0.05. †$0.05 \leq P$ (model) < 0.1. ‡P (likelihood ratio) < 0.05. §$0.05 \leq P$ (likelihood ratio) < 0.1.

Table 4—Performance of metabolite markers in the validation set

Metabolites	*P* value (Kruskal-Wallis test)	Fold change	
		DR cases vs. control subjects with diabetes	Control subjects with diabetes vs. individuals without diabetes
1,5-Gluconolactone	0.893	1.06	0.98
2-Deoxyribonic acid	0.014	1.27*	0.77
3,4-Dihydroxybutyric acid	0.029	1.39*	0.79
Erythritol	0.008	1.21*	0.99
Gluconic acid	<0.001	1.28*	1.45*
Lactose	0.543	1.05	1.08
Maltose	0.010	0.93	1.65*
Ribose	0.001	1.38*	0.91

*$P < 0.05$, Mann-Whitney *U* test.

medications (Table 1). These medications might have helped lower the magnitude of perturbations in amino acid metabolism than that found in untreated diabetes.

Nonreplicable Metabolite Markers of DR

Li et al. (26) investigated plasma metabolite markers of DR and found lowered levels of linoleic and arachidonic acids in DR cases. Although those findings are in agreement with the role of arachidonic acid being a precursor of proinflammatory eicosanoids (38), no significant differences between linoleic acid and arachidonic acid levels were found between DR cases and control subjects with diabetes in the discovery set (Supplementary Table 8). The levels of arachidonic acid are likely correlated to the use of antihypertensive medications rather than to DR status (39). This is supported by the similar numbers of participants on antihypertensive medications among the DR cases and control subjects with diabetes (27 and 24, respectively).

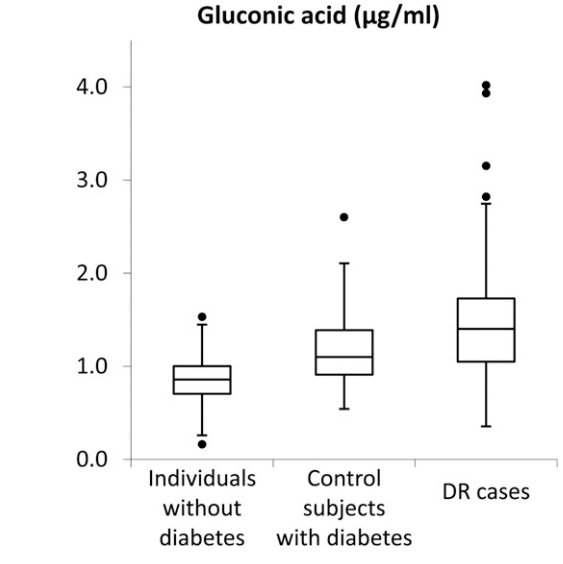

Figure 2—Plasma concentrations of gluconic acid from 40 individuals without diabetes, 40 control subjects with diabetes, and 40 DR cases in the validation set.

The n-3 polyunsaturated fatty acids (PUFAs) are thought to aid in diabetes prevention but meta-analyses on n-3 fatty acid intake show heterogeneous results, stratified by study locale (40). The n-3 PUFAs docosahexaenoic acid and eicosapentaenoic acid were detected on our platform, yet we did not find significant differences in these PUFAs between the DR group and control subjects with diabetes in the discovery set (Supplementary Table 8). Similarly, Mäkinen et al. (41) found that the baseline serum docosahexaenoic levels did not predict progressive kidney disease among patients with type 1 diabetes. Although n-3 PUFAs have been shown to protect against DR in rodent models (42,43), their efficacy in preventing vascular complications of diabetes can only be proven through randomized controlled trials.

Unique Signatures of Hydroxy Fatty Acids in DR

Earlier metabonomic studies on diabetes have demonstrated the association of several hydroxy fatty acids with the development of diabetes. 2-Hydroxybutyric acid, a derivative of α-ketobutyrate, was previously identified as a marker (14,21) and predictor (44) for diabetes. The ketone body, 3-hydroxybutyric acid, was identified as a marker for impaired glucose tolerance (13) and type 2 diabetes (20,21) and was also identified as a marker for DR in the study by Li et al. (26). No significant differences in the levels of 2-hydroxybutyric acid and 3-hydroxybutyric acid were found between DR cases and control subjects with diabetes in both the discovery and validation sets (Supplementary Table 8). Both DR cases and control subjects with diabetes showed increased levels of 2-hydroxybutyric acid compared with individuals without diabetes, consistent with a diagnosis of diabetes (Supplementary Fig. 2). These findings, together with aforementioned trends in amino acid levels, suggest that the plasma metabotype of DR is unique and not a mere extension of the plasma metabotype of diabetes.

There is accumulating evidence to support the contribution of altered gut microbiota to obesity and diabetes (45), and gut microbiota–derived short-chain fatty acids (acetate, propionate, and butyrate) have been identified as

signaling molecules in glucose and lipid metabolism (46). In our study, increased levels of 3,4-dihydroxybutyric acid and 2-deoxyribonic acid in DR cases were identified using discovery metabonomic profiling (Fig. 1 and Table 2). Similar increases in the concentrations of 3,4-dihydroxybutyric acid and 2-deoxyribonic acid were also found in the validation set (Table 4), though no significant differences in the levels of these hydroxy fatty acids were found between control subjects with diabetes and individuals without diabetes. There are no previous reports on the association of these hydroxy fatty acids with diabetes or DR. At present, it is known that 3,4-dihydroxybutyric acid is a urinary marker for succinate semialdehyde dehydrogenase deficiency (47) and the presence of 2-deoxyribonic acid within DNA is an indication of oxidative damage to the sugar moiety (48). These hydroxy fatty acids are not part of major metabolic pathways and may be products of gut microbial metabolism on butyrate or other short-chain fatty acids. Metabolic flux analysis of 2-deoxyribonic acid and 3,4-dihydroxybutyric acid could be performed to investigate their metabolic origins and their role in DR pathogenesis.

Metabonomic Profiling Identifies DR-Associated Pathways

Pathway mapping on MetaboAnalyst (37) showed that the pentose phosphate and galactose metabolism pathways are significantly enriched (FDR <0.2) among the metabolite markers identified from discovery metabonomic profiling. The pentose phosphate pathway was identified with increased levels of 1,5-gluconolactone; gluconic acid; and ribose, whereas galactose metabolism was identified with lactose, mannose, and *myo*-inositol (Supplementary Table 9). *Myo*-inositol levels were increased among DR cases in the discovery set (1.25-fold) but did not meet significance after correction for multiple testing. These results suggest that selected metabolite classes (organic acids, polyols and sugars, and oxidative stress markers) are involved in DR pathogenesis.

Erythritol is recommended as a diabetes-safe sweetener as it is a metabolically inert antioxidant (49). Earlier metabonomic studies have identified erythritol as a marker of diabetes (20) and impaired fasting glucose (15). In our study, DR cases in both the discovery and validation sets showed increased levels of erythritol when compared with control subjects with diabetes (Fig. 1, Table 2, and Table 4). In the absence of dietary survey data, it is unclear if this identified trend in erythritol levels was due to increased consumption of sweeteners or DR-related metabolic dysregulation. Mannose, a metabolite in galactose metabolism, was previously identified as a marker for impaired fasting glucose (14,15) and diabetes (14,20). Increased plasma levels of mannose were identified in DR cases from discovery metabonomic profiling (Fig. 1 and Table 2). Therefore, future studies targeting the quantitation of polyols and sugars become pertinent as metabolites in the pentose phosphate and galactose pathways are potential clinical metabolite markers of DR. Dietary information should also be incorporated in such studies to detect distinct identification of metabolite markers associated with DR resolved from potential confounding by related nutritional factors.

Increased polyol pathway flux is one of the main driving hypotheses responsible for diabetes vascular complications (50). Increased aldose reductase activity under hyperglycemic conditions consumes NADPH, affecting regeneration of reduced glutathione, thereby causing or worsening underlying oxidative stress. Although Barba et al. (24) established indirect evidence of increased polyol pathway flux in the analysis of vitreous humor of DR patients, increases in plasma levels of sorbitol and fructose, products of the polyol pathway, were not observed among participants with DR in this study. This is likely attributable to localization of aldose reductase activity on glucose in susceptible cell types—in this case, retina capillary endothelium (50)—and as a result, this perturbation remains undetected in plasma samples. The pentose phosphate pathway is the main source of cellular NADPH. Liu et al. (51) have identified activation of the pentose phosphate pathway in skeletal muscle mitochondria of adiponectin knockout mice on a high-fat diet, and these increases in pentose phosphate metabolites were associated with disturbances in glucose homeostasis and lipid metabolism. More recently, Grapov et al. (52) also identified increased plasma concentrations of gluconic acid in nonobese diabetic mice. The identification of the pentose phosphate pathway from discovery metabonomic profiling (Fig. 3) and the confirmation of increased gluconic acid (Fig. 2) and ribose levels in DR cases (Table 4) in the validation set demonstrate that oxidative stress is implicated in DR pathogenesis.

Limitations

Our study focused on the Indian (South Asian) ethnic group as the age-adjusted prevalence of diabetes in Indians is the highest among the major ethnic groups of Singapore (53). As cross-sectional sampling only captures a snapshot of plasma metabotypes, some of the identified markers may represent short-term metabolic perturbations instead of chronic risk factors associated with the development of DR. For example, discovery metabonomic profiling identified that 1,5-anhydroglucitol, a marker for short-term glycemic control (lowered values indicate hyperglycemia), was significantly lowered in DR cases with higher HbA_{1c} values than control subjects with diabetes. Adjustment for covariates in the logistic regression models might not be sufficiently robust, given the limited sample size. Although several metabolite markers were validated in an independent set where DR cases and control subjects with diabetes were matched for HbA_{1c}, the generalizability of these findings to other populations is unclear.

GC-MS is generally less sensitive than metabonomic profiling based on liquid chromatography–mass spectrometry and requires sample derivatization and elevated temperatures for analysis. Therefore, the list of identified metabolite

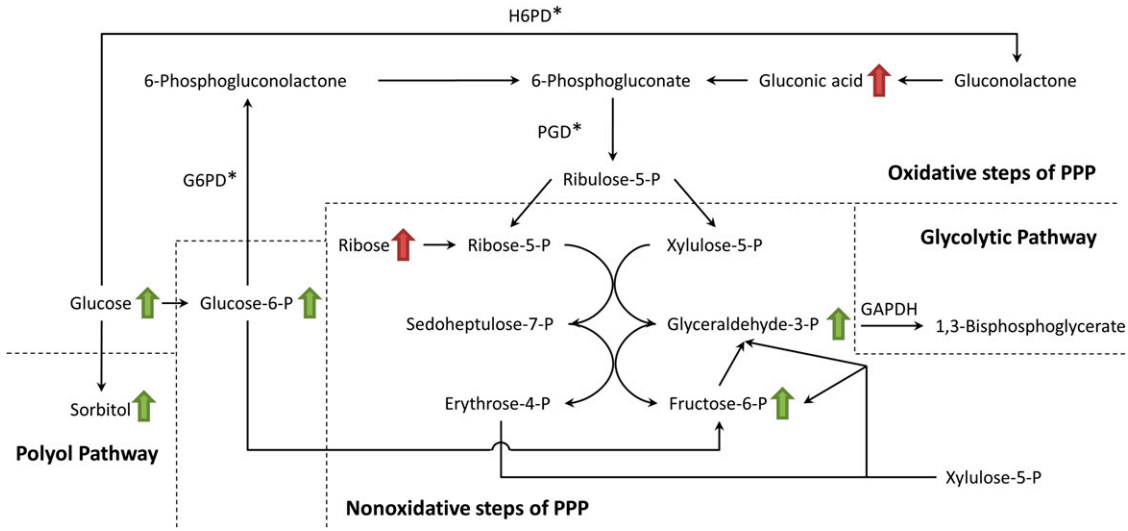

Figure 3—Activation of the pentose phosphate pathway (PPP) identified from elevated levels of gluconic acid and ribose (red arrows) among DR cases in both the discovery and validation sets. Reactions marked with an asterisk produce NADPH. Green arrows denote the accumulation of glycolytic metabolites leading to activation of the polyol, hexosamine, protein kinase C, and advanced glycosylation end product pathways in the unifying mechanism responsible for diabetes complications, as proposed by Brownlee (50). G6PD, glucose-6-phosphate dehydrogenase; H6PD, hexose-6-phosphate dehydrogenase; P, phosphate; PGD, phosphogluconate dehydrogenase.

markers in our study could be limited to compounds that form stable silylated derivatives.

Conclusions

Using global metabonomic profiling, this study has identified plasma metabolite signatures that distinguish patients with diabetes and retinopathy from those without retinopathy. These metabolite markers remained significant and robust while controlling for established risk factors. The metabolite markers 2-deoxyribonic acid; 3,4-dihydroxybutyric acid; erythritol; gluconic acid; and ribose were validated in an independent, HbA_{1c}-matched sample set. 2-Deoxyribonic acid and 3,4-dihydroxybutyric acid are novel metabolite markers with no prior reports of association with diabetes or DR. The pentose phosphate pathway was identified as a key metabolic dysregulation associated with DR, demonstrating the involvement of oxidative stress in disease pathogenesis. These findings provide the foundation for longitudinal metabonomic studies to establish the correlation and predictive value of metabolite profiles with the rate of DR progression in patients with diabetes.

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