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Circulating metabolomic markers in association with overall burden of microvascular complications in type 1 diabetes

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ABSTRACT

Introduction Diabetic retinopathy (DR), diabetic kidney disease (DKD) and distal symmetric polyneuropathy (DSPN) share common pathophysiology and pose an additive risk of early mortality.

Research design and methods In adults with type 1 diabetes, 49 metabolites previously associated with either DR or DKD were assessed in relation to presence of DSPN. Metabolites overlapping in significance with presence of all three complications were assessed in relation to microvascular burden severity (additive number of complications—ie, presence of DKD±DR±DSPN) using linear regression models. Subsequently, the same metabolites were assessed with progression to endpoints: soft microvascular events (progression in albuminuria grade, ≥30% estimated glomerular filtration rate (eGFR) decline, or any progression in DR grade), hard microvascular events (progression to proliferative DR, chronic kidney failure, or ≥40% eGFR decline), and hard microvascular or macrovascular events (hard microvascular events, cardiovascular events (myocardial infarction, stroke, or arterial interventions), or cardiovascular mortality), using Cox models. All models were adjusted for sex, baseline age, diabetes duration, systolic blood pressure, HbA1c, body mass index, total cholesterol, smoking, and statin treatment.

Results The full cohort investigated consisted of 487 participants. Mean (SD) follow-up was 4.8 (2.9, 5.7) years. Baseline biothesiometry was available in 202 participants, comprising the cross-sectional cohort. Eight metabolites were significantly associated with presence of DR, DKD, and DSPN, and six with additive microvascular burden severity. In the full cohort longitudinal analysis, higher levels of 3,4-dihydroxybutanoic acid (DHBA), 2,4-DHBA, ribonic acid, glycine, and ribitol were associated with development of events in both crude and adjusted models. Adding 3,4-DHBA, ribonic acid, and glycine to a traditional risk factor model improved the discrimination of hard microvascular events.

Conclusions While prospective studies directly assessing the predictive ability of these markers are needed, our results strengthen the role of clinical metabolomics in relation to risk assessment of diabetic complications in chronic type 1 diabetes.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Several circulating metabolites have been associated with the presence and progression of diabetic retinopathy and diabetic kidney disease. But information regarding the role of circulating metabolites and diabetic neuropathy and overall complication burden on people with type 1 diabetes is lacking.

WHAT THIS STUDY ADDS

⇒ We have investigated a panel of circulating metabolites with increasing microvascular complication burden in type 1 diabetes, and thereafter assessed relevant metabolites with progression of microvascular and macrovascular diseases. We identified four metabolites, 3,4-dihydroxybutanoic acid, 2,4-dihydroxybutanoic acid, ribonic acid, glycine, and ribitol, each associated with increasing microvascular burden, as well as associated with the development of microvascular and macrovascular outcomes.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Clinical metabolomics is increasingly being demonstrated as a valid tool for risk assessment of diabetic complications in type 1 diabetes.

INTRODUCTION

The search for new and improved methods for assessing the risk of complications in type 1 diabetes (T1D) is ever ongoing. People commonly develop T1D at an early stage in life resulting in many years of high risk of complications. Diabetic retinopathy (DR), kidney disease, neuropathy, and cardiovascular disease are all frequent and highly debilitating conditions leading to reduced life expectancy and lower quality of life.[1 2] In the shadow of the much greater global issue of type 2 diabetes (T2D),[3] research and

treatment of complications in T1D often rely on data collected in people with T2D, despite a large pathophysiological and phenotypic division. Thus, investigating the interplay and overlap between different complications in T1D accurately is highly relevant in order to target appropriate interventions at an earlier stage of complication development.

We have previously investigated the associations between circulating metabolites and lipid species and diabetic complications in T1D, both cross-sectionally and prospectively.[4–9] Through these studies, we have identified hydroxybutyrate isomers, ribose derivatives, and branched-chain amino acids that were associated with presence and development or progression of DR,[8] kidney disease,[5] and cardiovascular disease.[10] Similar results were also identified in relation to cardiac autonomic neuropathy.[4 11] Distal symmetric polyneuropathy (DSPN) remains to be investigated using similar methods. Further, identification of common metabolic risk predictors for multiple microvascular and/or macrovascular complications or '*burden of complications*' in individuals with T1D is of major importance.

In this study, we set out to investigate the role of 49 circulating metabolites previously related to chronic kidney disease (CKD) and DR in relation to the presence of DSPN in T1D, in order to investigate the role of shared metabolites across overall burden of complications, cross-sectionally and longitudinally.

METHODS

Through 2009–2011, a total of 667 individuals with T1D exhibiting albuminuria, ranging from normal to severely increased, were recruited from Steno Diabetes Center Copenhagen. Individuals with kidney failure were excluded. Details of the cohort have been described previously.[12] Of these, 258 participants were included in a substudy investigating autonomic neuropathy in which measures of DSPN and cardiac autonomic neuropathy were performed.[4] The present study includes 487 participants from the original cohort in which data on DR, diabetic kidney disease (DKD), and cardiovascular disease were available, of which 202 have data on DSPN available and were included in cross-sectional analyses. All participants gave written informed consent, and the study was conducted in accordance with the Declaration of Helsinki.

Baseline data

Venous sampling analyzed by standardized methods was employed to quantify serum creatinine, glycated hemoglobin (HbA1c), cholesterol, and triglycerides. Urinary albumin excretion rate (UAER) was quantified using the mean of three consecutive 24-hour urine collections and analyzed by enzyme immunoassay. The Chronic Kidney Disease Epidemiology Collaboration 2009 equation[13] was employed to calculate estimated glomerular filtration rate (eGFR). A validated automated sphygmomanometer was used to assess sitting brachial blood pressure after 10 min of rest.

Presence of DSPN was defined by biothesiometry vibration perception test above an individualized threshold, bilaterally, adjusted for age, height, and sex using the following algorithm[14 15]:

$$exp\left(-0.51+\left(0.0248\times age\right)+\left(0.77\times height\ (m)\right)+\left(0.03\times sex\right)+s\right),$$

where s=SD of expected log vibration threshold (s=0.62) and sex is defined as 1 for men and 2 for women.

Baseline normal, moderately, and severely increased albuminuria were defined as UAER <30 mg/24 hours, 30–299 mg/24 hours, and >299 mg/24 hours, respectively, in two out of three consecutive 24-hour urine collections. Presence of CKD was based on the Kidney Disease Improving Global Outcomes 2012 Guideline[16] categories in which eGFR <60 mL/min/1.73 m^2 or UAER ≥30 mg/24 hours or urinary albumin creatinine rate (UACR) >30 mg/g is considered manifest CKD. DR measurements have been described previously.[8] In short, DR was classified using a 0–4 in-house algorithm which, in turn, is based on five mydriatic non-stereoscopic retinal fundus photos combined into a mosaic, graded using a modified version of the International Classification of Diabetic Retinopathy Disease Severity Scale.[17] DR, according to this scale, is defined as no DR, mild non-proliferative, moderate non-proliferative, proliferative, and proliferative DR with fibrosis. Blind participants were excluded. Subsequently, it was subdivided into low-grade DR defined as no or mild non-proliferative DR, and high-grade DR including moderate non-proliferative and proliferative DR. Finally, by adding the number of microvascular complications (presence of DKD+DR+DSPN) prevalent in each participant, an overall score representing microvascular complication burden was calculated (ie, no complication=0, one complication=1, up to a maximum burden score of 3).

Serum metabolomics: measurements and selection

Metabolomic sample analysis has been described previously.[5] In short, serum samples stored at −80°C were analyzed using two-dimensional gas chromatography with time-of-flight mass spectrometry. Raw data were processed by peak picking using ChromaTOF and thereafter aligned using Guineu.[18] Subsequently, data were postprocessed in R. All metabolites associated ($p<0.05$) with diabetes microvascular complications of the kidney (eGFR and albuminuria) or DR score within the same T1D cohort from Steno Diabetes Center Copenhagen, published previously,[5 8] were selected for investigating the association with DSPN. Selected metabolites (n=49) are listed in online supplemental table 1.

Follow-up

Follow-up information on kidney disease, cardiovascular disease, retinopathy, and clinical measurements from inclusion in 2009–2011 until December 31, 2016 was obtained for all participants. All participants were also

traced through the Danish National Death Register until December 31, 2015 to obtain data on mortality, including information on date and cause of death. Information regarding kidney disease and cardiovascular disease was sourced through the Danish National Health Register. Registry data on biothesiometry, or other measures of peripheral autonomic neuropathy, were not available and could therefore not be assessed during follow-up. Retinopathy measurements were extracted from local electronic records from Steno Diabetes Center Copenhagen. UAER, UACR, serum creatinine, systolic blood pressure and HbA1c measurements were extracted from standard ambulatory care records.

Definition of longitudinal composite endpoints

Events were classified into three combined endpoints as well as into two categories. The first category was defined as 'soft' endpoints (broader and earlier events using less established disease stages). The second category was defined as 'hard' outcomes (more robustly characterized events or disease stages). The first combined endpoint, soft microvascular events, included any progression in retinopathy status from any lower to any higher/more advanced DR stage as described above; any progression in albuminuria from normal or moderately increased albuminuria to moderately or severely increased albuminuria; or ≥30% decline in eGFR from baseline. The second endpoint, hard microvascular events, included progression from no or non-proliferative DR to proliferative DR; development of chronic kidney failure (defined as CKD grade 5, chronic dialysis, kidney transplantation, or eGFR <15 mL/min/1.73 m^2); or ≥40% decline in eGFR from baseline. The last endpoint combines the hard microvascular events with development of cardiovascular events (defined as non-fatal myocardial infarction, non-fatal stroke, coronary interventions, or peripheral arterial interventions) or cardiovascular mortality. Diagnosis codes and procedural codes used to trace endpoints can be found in online supplemental table 2. Registry data on neuropathy were not available and therefore not included in the composite endpoints.

Statistical analyses

Normally distributed continuous variables are shown as mean±SD, non-normally distributed as median (IQR), and categorical variables as n (%). Before inclusion in models, non-normal distributed variables were log2 transformed. At baseline, metabolites previously shown to be significantly associated with baseline DR score, eGFR, and albuminuria grade,[5 8] using the same metabolomic panel, were investigated using linear regression models in association with baseline presence of DSPN. Thereafter, metabolites overlapping in significance (p<0.05) with the presence of all above-mentioned microvascular complications were included in the analysis for overall complication burden.

Overlapping metabolites (online supplemental figure 1) were then investigated in association with baseline microvascular complication burden using crude and multivariate linear regression models. Included covariates were age, sex, diabetes duration, systolic blood pressure, HbA1c, body mass index, total cholesterol, current smoking, and statin treatment. Estimates are presented as log2 fold change (logFC) of the metabolites.

Overlapping metabolites from the cross-sectional analysis were then assessed in the longitudinal cohort in association with time to first event endpoints using crude and multivariate adjusted Cox proportional hazards models. If multiple endpoints in the composite were achieved, only the first was analyzed. Adjustment included the above-mentioned clinical covariates, as well as baseline eGFR and UAER. HRs are presented with 95% CIs reflecting the risk of outcome for a metabolite level above the median compared with a level below.

Finally, the discrimination potential of metabolites shown to be significantly associated to endpoint development in Cox proportional hazards models was calculated using C-statistics. Receiver operating characteristic (ROC) curves including the metabolites alone, with clinical covariates (the same as for the Cox proportional hazards models), and clinical covariates alone, were drawn, and areas under the curve (AUCs) were calculated. AUCs of ROC curves including metabolites with clinical covariates, and clinical covariates alone, were tested against each other for significance using Z test.

All statistical analyses and data visualizations were performed using R (V.4.2.1, R Core Team, Vienna, Austria) in RStudio (V.2022.07.2+576, RStudio Team, Boston, Massachusetts, USA). A two-sided p value <0.05 was considered significant.

RESULTS

Baseline characteristics

Of the 487 participants, 261 (54%) were male. At baseline, mean±SD age was 55±12 years, diabetes duration was 32±16 years, and HbA1c was 65±13 mmol/mol (8.1±1.2%). The subcohort with DSPN measures, including 202 participants, was reflective of the entire cohort with only minor differences. DSPN was present at baseline in 155 (77%) participants. All baseline information can be seen in table 1. A flow chart describing the selection process of the participant cohorts is presented in online supplemental figure 2.

Cross-sectional associations with DSPN

At baseline, nine metabolites were associated with the presence of DSPN (online supplemental figure 3). Higher levels of 3,4-dihydroxybutanoic acid (DHBA) and 2,4-DHBA were both associated with the presence of DSPN (logFC=0.30 (95% CI 0.12, 0.48) and 0.37 (95% CI 0.20, 0.55), respectively, p<0.001) as well as another hydroxy fatty acid, 2-hydroxyisovaleric acid, although with a weaker, inverse association (logFC=−0.16 (95% CI 0.28, −0.05), p=0.006). Higher levels of the sugar derivates myoinositol, ribitol, and ribonic acid were associated with

Table 1 Baseline characteristics of both the subcohorts, including participants with biothesiometry measures, and the full cohort in which longitudinal endpoints were investigated

Variables	DSPN cohort	Full cohort
N	202	487
Age, years	57 (50, 64)	55 (12)
Men, n (%)	97 (48)	261 (54)
Diabetes duration, years	41 (9)	32 (16)
Body mass index, kg/m^2	24.7 (3.8)	25.4 (6.4)
HbA1c, mmol/mol	63 (11)	65 (13)
HbA1c, %	7.9 (1.0)	8.1 (1.2)
Systolic blood pressure, mm Hg	134 (14)	132 (17)
Total cholesterol, mmol/L	4.57 (0.81)	4.70 (0.86)
Low-density lipoprotein cholesterol, mmol/L	2.34 (0.67)	2.48 (0.75)
eGFR, mL/min/1.73 m^2	78 (26)	82 (25)
Urinary albumin excretion rate, mg/24 hours	14 (6, 54)	17 (8, 57)
Statin treatment, n (%)	126 (62)	294 (60)
RAASi treatment, n (%)	202 (100)	332 (68)
Current smoker, n (%)	31 (15)	99 (20)
Retinopathy grade, n (%)		
No retinopathy	16 (8)	106 (22)
Mild non-proliferative	32 (16)	68 (14)
Moderate to moderate-severe non-proliferative	64 (32)	147 (30)
Proliferative	45 (22)	91 (19)
Proliferative with fibrosis	45 (22)	75 (15)
DSPN, n (%)	155 (77)	–

Continuous variables are presented as mean (SD) if normally distributed, or median (IQR) if non-normally distributed. Categorical variables are presented as n (%).
DSPN, distal symmetric polyneuropathy; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; RAASi, renin-angiotensin-aldosterone system inhibitor.

presence of DSPN (online supplemental table 3). The three amino acids valine, glycine, and leucine were associated with DSPN; glycine being positively associated, and valine and leucine negatively associated.

Cross-sectional associations with microvascular complication burden

Eight overlapping metabolites were associated with DSPN, DR, and DKD at baseline (online supplemental figure 1). All eight metabolites were also associated with the microvascular burden score (p≤0.028). 3,4-DHBA and 2,4-DHBA exhibited the largest logFCs (0.15 (0.08, 0.23), p<0.001; and 0.18 (0.11, 0.25), p<0.001) (table 2). After adjustment, 2-hydroxyvaleric acid and glycine lost significance. However, all other metabolites remained significantly associated with higher microvascular complication burden (table 2, figure 1).

Table 2 Association between baseline metabolite levels (estimates are presented as log2 fold change (logFC)) and microvascular complication burden (defined as: no complication=0, one complication=1, up to a maximum burden score of 3)

Metabolite	LogFC	95% CI	P value
2,4-Dihydroxybutanoic acid	0.32	0.15, 0.50	<0.001
Ribonic acid	0.31	0.14, 0.48	<0.001
Myoinositol	0.28	0.12, 0.44	<0.001
Ribitol	0.29	0.11, 0.47	0.002
3,4-Dihydroxybutanoic acid	0.26	0.07, 0.45	0.007
Valine	−0.16	−0.32, −0.01	0.037
Glycine	0.10	−0.05, 0.25	0.202
2-Hydroxyisovaleric acid	0.00	−0.11, 0.12	0.969

Estimates, 95% CIs, and p values were calculated using linear regression models adjusted for age, sex, diabetes duration, systolic blood pressure, glycated hemoglobin, body mass index, total cholesterol, smoking, and statin treatment.

Longitudinal associations with progression of complications

Follow-up was median 4.8 (2.9, 5.7) years. There were 211 (43%) people experiencing a soft microvascular event, 107 (22%) a hard microvascular event, and 140 (29%) a hard microvascular and macrovascular event, respectively.

The longitudinal analyses of the eight metabolites associated with all three complications at baseline (table 3, online supplemental table 4, and figure 2) showed that levels of 3,4-DHBA above the median, when compared with levels below, were associated with all three composite endpoints after adjustment. The association with hard microvascular events was strongest (HR (95% CI): 2.02 (1.18, 3.45), p=0.011). In contrast, 2,4-DHBA exhibited significant associations with both microvascular endpoints (soft: 1.41 (1.03, 1.92), p=0.031; hard: 1.83 (1.11, 3.02), p=0.018), but not with the combined microvascular and macrovascular endpoints. Glycine and ribonic acid levels above the median were not associated with the soft microvascular endpoint when compared with levels below, but with both the hard microvascular (glycine: 1.58 (1.04, 2.40), p=0.031; ribonic acid: 1.91 (1.18, 3.12), p=0.009) and the combined endpoint (glycine: 1.53 (1.06, 2.20), p=0.023; ribonic acid: 1.55 (1.03, 2.33), p=0.035). Finally, ribitol levels above the median were associated with higher risk of developing the combined endpoint

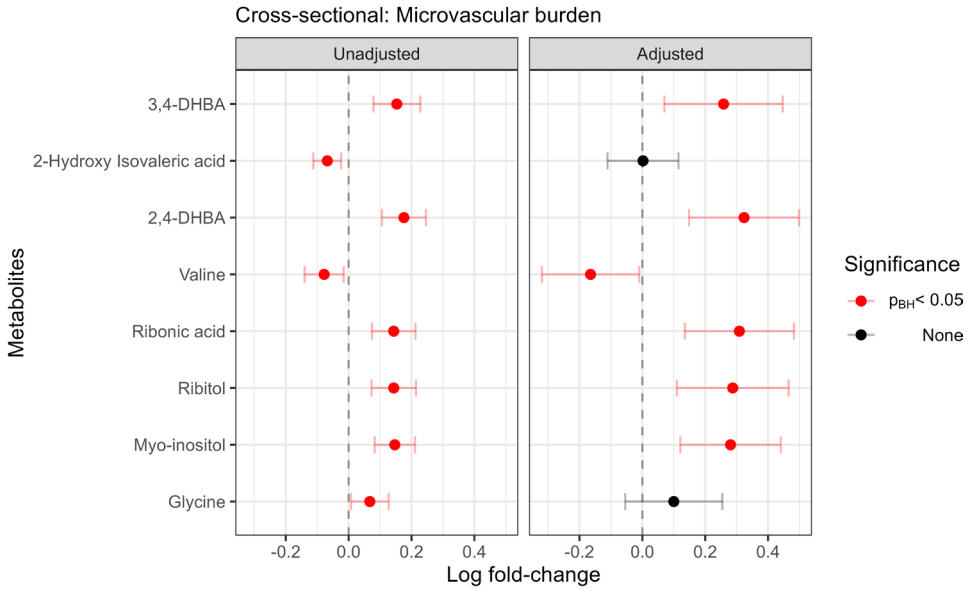

Figure 1 Forest plot illustrating the log2 fold increase estimates and 95% CIs of metabolites exhibiting significance in relation to microvascular complication burden in linear regression models adjusted for age, sex, diabetes duration, HbA1c, systolic blood pressure, total cholesterol, body mass index (BMI), and statin treatment. DHBA, dihydroxybutanoic acid.

compared with levels below the median (1.59 (1.04, 2.42), p=0.033).

C-statistics

The best performing metabolites in longitudinal models (in terms of exhibited significance), 3,4-DHBA, 2,4-DHBA, ribonic acid, and glycine, were selected for investigation of their discriminatory potential using C-statistics. Discrimination models were built including the same variables used for adjusting linear regression and Cox proportional hazards models, as previously described. Endpoints investigated using C-statistics were chosen based on the results from the Cox proportional hazards models presented in table 3. Metabolites significantly associated with increased risk of an endpoint in Cox proportional hazards models were investigated in relation to the same endpoint using C-statistics. AUCs for the single metabolites ranged from 0.498 to 0.752 (online supplemental table 5). Thereafter, AUCs were

Table 3 HRs of the association between baseline metabolite levels and soft microvascular (DR progression, albuminuria progression, or ≥30% estimated glomerular filtration rate (eGFR) decline), hard microvascular (proliferative DR, kidney failure, or ≥40% eGFR decline), and hard microvascular and macrovascular (hard microvascular events, cardiovascular events, or cardiovascular mortality)

	Microvascular—soft n=211		Microvascular—hard n=107		Microvascular and macrovascular—hard n=140	
Metabolite	**HR (95% CI)**	**P value**	**HR (95% CI)**	**P value**	**HR (95% CI)**	**P value**
2-Hydroxyisovaleric acid	0.97 (0.74, 1.29)	0.851	0.95 (0.64, 1.42)	0.808	1.06 (0.75, 1.50)	0.810
3,4-Dihydroxybutanoic acid	1.39 (1.01, 1.92)	**0.042**	2.02 (1.18, 3.45)	**0.011**	1.70 (1.09, 2.67)	**0.021**
2,4-Dihydroxybutanoic acid	1.41 (1.03, 1.92)	**0.031**	1.83 (1.11, 3.02)	**0.018**	1.48 (0.97, 2.25)	0.068
Glycine	1.16 (0.88, 1.55)	0.291	1.58 (1.04, 2.40)	**0.031**	1.53 (1.06, 2.20)	**0.023**
Myoinositol	1.16 (0.83, 1.62)	0.385	1.41 (0.92, 2.40)	0.202	1.45 (0.92, 2.28)	0.108
Valine	0.96 (0.73, 1.28)	0.800	0.68 (0.45, 1.02)	0.064	0.82 (0.58, 1.16)	0.255
Ribitol	1.10 (0.81, 1.51)	0.540	1.39 (0.85, 2.28)	0.189	1.59 (1.04, 2.42)	**0.033**
Ribonic acid	1.32 (0.97, 1.79)	0.077	1.91 (1.18, 3.12)	**0.009**	1.55 (1.03, 2.33)	**0.035**

HRs reflect the risk of outcome for a metabolite level above the median compared with below the median. HRs, 95% CIs, and p values were calculated using Cox proportional hazards models adjusted for sex, baseline age, diabetes duration, systolic blood pressure, glycated hemoglobin, body mass index, total cholesterol, smoking, statin treatment, eGFR, and urinary albumin excretion rate. Significant associations are highlighted by p values in bold font.
DR, diabetic retinopathy.

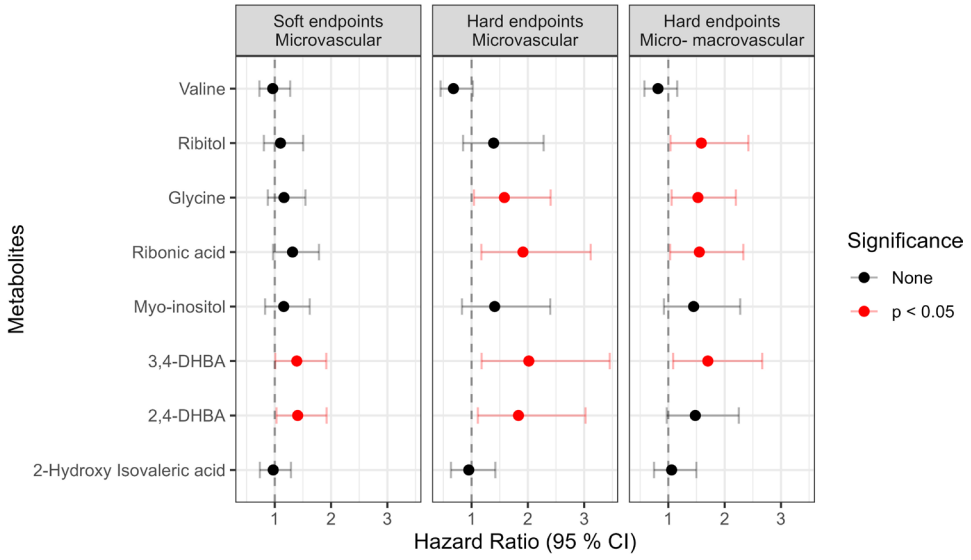

Figure 2 Forest plot illustrating the adjusted HRs and 95% CIs for investigated metabolites in relation to soft microvascular, hard microvascular, and hard microvascular and macrovascular endpoints. HRs reflect the risk of outcome for a metabolite level above the median compared with below the median. Adjustment included age, sex, diabetes duration, HbA1c, systolic blood pressure, estimated glomerular filtration rate, urinary albumin excretion rate, total cholesterol, body mass index (BMI), and statin treatment. DHBA, dihydroxybutanoic acid.

calculated adding multiple metabolites to assess potential discrimination improvement. 3,4-DHBA, ribonic acid, and glycine, in addition to the traditional risk factor model versus the traditional risk factor model alone, significantly improved the discrimination of hard microvascular events (AUC: 0.840 vs 0.818, p=0.047, online supplemental figure 4). The other comparisons did not exhibit significance.

DISCUSSION

In the present study, we reported that several circulating metabolites are associated with higher microvascular burden at baseline and subsequently higher risk of development of both microvascular and macrovascular complications. Specifically, 3,4-DHBA (also known as protocatechuic acid) was associated with all complications and endpoints, independent of traditional risk factors, identifying it as possible novel risk markers for complications in T1D. Several other metabolites were likewise associated with higher risk of developing complications, but not uniformly so; with ribonic acid and glycine exhibiting particularly robust significance with the hard composite endpoints. Finally, in combination with previous work, we showed that various circulating metabolites in individuals with T1D seem to be valid risk markers of both complication burden and development.

Previous exploratory studies, using the same cohort, have demonstrated that a metabolomic approach for risk stratification of diabetic complications is both feasible and independent of traditional risk factors.[4 5 8 10] However, due to the large heterogeneity across metabolomic panels and platforms used for various studies, the metabolites identified are difficult to validate. Higher 3,4-DHBA levels have been associated with presence of DR in a cross-sectional study of 80 individuals with T2D as well as 2,3-DHBA being associated with an increased risk of severely increased albuminuria in T1D,[19] but no others have shown the same associations with diabetic complications in humans. In a study investigating the effect on exercise intervention on the metabolome in rats with streptozocin-induced diabetes, higher ribonic acid and lower 2,3-DHBA levels were found in diabetic versus control rats, as well as a decrease in ribonic acid and an increase in 2,3-DHBA after the intervention in the diabetic model. This suggests that ribonic acid and DHBAs might serve as modifiable markers for improved monitoring of treatment or prevention[20] of microvascular complications in T1D.

Glycine, in turn, is relatively more established as a marker of hyperglycemia, obesity, metabolic dysfunction, and risk of vascular complications in diabetes and non-diabetes alike.[21–24] Already in 1985, a study indicated a higher glycine synthesis in a hyperglycemic versus a normoglycemic state.[25] Subsequently, insulin treatment normalizes increased branched-chain amino acid excretion levels in newly diagnosed T1D individuals.[26] More recent results have specifically implied glycine's role as a marker of DKD. A study of 52 Finnish individuals with T1D with normal urinary albumin excretion identified acylglycines, as well as acylcarnitines, and components of tryptophan metabolism to be discriminatory of onset of albuminuria.[27] Moreover, another study investigating a murine DKD model showed that glycine metabolism in serum as well as kidney tissue was altered by empagliflozin, a sodium-glucose cotransporter 2 inhibitor,[28] further implying glycine as a marker in kidney disease pathophysiology. We used a metabolomic panel developed in house which identifies 75 circulating serum metabolites,[5]

a fraction of all active circulating metabolites. A recent systematic review on metabolomics in DR[29] demonstrates several metabolites of interest, associated with the presence of DR in plasma, as well as aqueous and vitreous humor; the latter ones assessing local changes in the metabolome, compared with investigating metabolites in circulation. Methods of investigating the metabolomes and proteomes of renal, cardiac, or neural tissues may similarly entail local active metabolites that could have more precise prognostic ability. However, these methods are generally more impractical than plasma or serum metabolomics derived from standard venipuncture.

Our results demonstrate a feasible approach using circulating metabolomics for better stratification of risk for complications in T1D. Additionally, we demonstrate an association with development of microvascular and macrovascular endpoints, as well as show an improvement in the discrimination of development of hard microvascular endpoints. However, observational trials are needed to investigate the prospective discrimination of risk with these markers, as well as clinical intervention trials targeting or measuring DHBAs and amino acids, such as glycine, in relation to key clinical targets in diabetic complications. Recommended screening for diabetic microvascular complications includes yearly assessment of albuminuria and kidney function, periodic fundus photography, and periodic assessment of diabetic peripheral and autonomic neuropathy.[30 31] Metabolomic analyses on top of existing screening guidelines may highlight individuals at highest risk of progression to be able to target resources toward where they are most needed. However, while the metabolites identified in this paper are associated with complications in T1D, T1D and T2D have differing phenotypes and risk profiles to development of diabetic complications. Further studies are needed to elucidate their role in T2D; particularly given the marked heterogeneity of T2D.[32] There are several limiting factors in our study. Mainly, we lack an independent cohort in which to validate our results—but we would like to argue that our findings are in line with previous evidence and thus we do consider our results to be applicable, all while acknowledging that any conclusion should be taken with care. Furthermore, there is limited baseline information in the present cohort regarding lifestyle parameters, socioeconomic information, and concomitant medication, prohibiting addressing these factors in our analyses. Likewise, our baseline cross-sectional population was relatively small due to only a selected portion of the full cohort having had biothesiometry performed (n=202), which could also influence our results. Similarly, follow-up information from this cohort has been extracted from national registries which are, although validated by many studies before, inherently limiting compared with observational studies examining participants at specific time points, collecting standardized data. Therefore, no information on biothesiometry, or other measures of peripheral autonomic neuropathy, could be included in the longitudinal part of this study, thus severely impairing the applicability of our findings to neuropathic risk stratification. Additionally, the associations demonstrated in this study, despite being adjusted for multiple traditional risk factors, may still be subject to a number of confounding factors. This study cannot conclusively attest to the identified metabolites being causally related to higher risk of complications; further studies, specifically designed to investigate the causal effect of elevated circulating metabolites, are needed in order to determine causality. Finally, our population was included from a single tertiary diabetes center and the cohort composed of almost exclusively white individuals. The results presented in this paper may differ across T1D individuals of other populations and ethnicities.

CONCLUSIONS

We have identified novel metabolites in plasma to be associated with DSPN in T1D. Building on these and previous findings, we have further selected eight metabolites which are associated with increased microvascular complication burden in T1D. Of these, 3,4-DHBA, ribonic acid, glycine, and 2,4-DHBA were significantly associated with prospective development of both soft and hard microvascular complications; and three of these, 3,4-DHBA, ribonic acid, and glycine, improved the predictive discrimination of development of hard microvascular endpoints. Finally, higher levels of 3,4-DHBA, ribonic acid, glycine, and ribitol were significantly associated with development of a composite of both microvascular and macrovascular events. While prospective studies directly assessing the predictive ability of these markers are needed, our results strengthen the role of clinical metabolomics in relation to risk assessment of diabetic complications in chronic T1D.

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Contributors VRC wrote the first draft. VRC and BAS performed the statistical analyses. VRC, BAS, TWH, PR, and TSA designed the study. VRC, BAS, TWH, SYJ, KS, IMM, MF-M, KT, CL-Q, ST, NT, SAW, CSH, PR, and TSA were involved in data collection and interpretation. TSA supervised the study. All authors revised the draft manuscript and approved the submission for publication. TSA is responsible for the work as a whole.

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