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Blood Metabolomic Measures Associate With Present and Future Glycemic Control in Type 2 Diabetes

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Objective: We studied whether blood metabolomic measures in people with type 2 diabetes (T2D) are associated with insufficient glycemic control and whether this association is influenced differentially by various diabetes drugs. We then tested whether the same metabolomic profiles were associated with the initiation of insulin therapy.

Methods: A total of 162 metabolomic measures were analyzed using a nuclear magnetic resonance-based method in people with T2D from four cohort studies (n = 2641) and one replication cohort (n = 395). Linear and logistic regression analyses with adjustment for potential confounders, followed by meta-analyses, were performed to analyze associations with hemoglobin A1c levels, six glucose-lowering drug categories, and insulin initiation during a 7-year follow-up period (n = 698).

Results: After Bonferroni correction, 26 measures were associated with insufficient glycemic control (HbA1c >53 mmol/mol). The strongest association was with glutamine (OR, 0.66; 95% CI,

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Abbreviations: Apo, apolipoprotein; BCAA, branched chain amino acid; BMI, body mass index; CODAM, Cohort on Diabetes and Atherosclerosis Maastricht; DCS, Hoorn Diabetes Care System cohort study; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NEO, the Netherlands Epidemiology of Obesity study; T2D, type 2 diabetes; VLDL, very-low-density lipoprotein.

0.61 to 0.73; $P = 7.6 \times 10^{-19}$). In addition, compared with treatment-naive patients, 31 metabolomic measures were associated with glucose-lowering drug use (representing various metabolite categories; $P \leq 3.1 \times 10^{-4}$ for all). In drug-stratified analyses, associations with insufficient glycemic control were only mildly affected by different glucose-lowering drugs. Five of the 26 metabolomic measures (apolipoprotein A1 and medium high-density lipoprotein subclasses) were also associated with insulin initiation during follow-up in both discovery and replication. The strongest association was observed for medium high-density lipoprotein cholesteryl ester (OR, 0.54; 95% CI, 0.42 to 0.71; $P = 4.5 \times 10^{-6}$).

Conclusion: Blood metabolomic measures were associated with present and future glycemic control and might thus provide relevant cues to identify those at increased risk of treatment failure. (***J Clin Endocrinol Metab*** **103: 4569–4579, 2018)**

Type 2 diabetes (T2D) is a very heterogeneous disease, which is also reflected in the heterogeneity in response to glucose-lowering treatment. Previously, we showed distinct trajectories of glucose control in people with T2D, with most achieving good glycemic control (1). People with T2D who are not treated optimally have an increased risk of developing diabetes-related complications (1, 2). As such, interest has been increasing to discover the factors associated with a poor treatment response to facilitate personalized therapeutics.

Recent technologic advances have allowed for the simultaneous detection of a wide range of metabolites in biological samples to acquire information on multiple pathways relevant for a person's metabolic state (3). The rapid developments in technology to determine a blood metabolomic profile combined with highly standardized, reproducible, and affordable measurements could facilitate the introduction of metabolomics in daily clinical practice with the aim of advancing the personalization and effectiveness of the treatment of T2D.

Blood metabolomic measures such as branched chain amino acids (BCAAs), α-hydroxybutyrate, 2-aminoadipic acid, various lipids, and other metabolites have been associated with the risk of T2D (4–6). Changes in the blood metabolomic profile might reflect early changes in the disease process of T2D but might also influence diabetes progression. As such, metabolomics could be a useful tool in the early identification and stratification of those at increased risk of T2D and to acquire knowledge about the disease etiology and progression (4). Although previous findings have shown that metabolomic profiles provide information in addition to the well-known clinical risk factors in the prediction of the development of T2D (7), only a few studies have investigated their utility in the assessment of treatment response and disease progression. These studies mostly investigated which metabolites respond to the initiation of glucose-lowering drugs (8, 9); however, they were often limited to only a single drug and small patient cohorts.

In search of better markers of a successful treatment response, we used the metabolomic data from four independent T2D cohorts from the Netherlands. The metabolomic measures investigated belong to several classes, including amino acids, glycolysis measures, ketone bodies and fatty acids, and the lipid concentrations and compositions of 14 lipoprotein subclasses. We assessed the cross-sectional and glucose-lowering drug-stratified associations of these metabolomic measures with glycemic control. Three cohorts provided data to examine the prospective association of metabolomic measures with diabetes progression.

Materials and Methods

T2D cohorts

Data from patients with T2D (n = 2641) from four different cohorts from the Netherlands were used; the Hoorn Diabetes Care System cohort study (DCS; n = 995) (10), the Maastricht study (n = 848) (11), the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM; n = 134) (12), and the Netherlands Epidemiology of Obesity study (NEO; n = 664) (13). Prospective data from follow-up visits were available from two studies (DCS and CODAM; n = 698) and from an independent replication study, the Rotterdam study (n = 395) (14). All studies were conducted in accordance with the Declaration of Helsinki and approved by the relevant local medical ethics committees. All participants have provided written informed consent before entering the study. Detailed cohort descriptions and study characteristics are described in the subsequent paragraphs (Table 1; Supplemental Tables 1–5).

DCS cohort study

The DCS provides routine diabetes care to patients living in the West-Friesland region (10). Patients visit the DCS research center annually, during which blood samples are taken with the patient in the fasting state for routine biochemistry tests. The patients also receive a full medical examination, advice about their health and treatment, and education about their disease during their annual visits to the DCS research center. In addition, patients are invited to join our research and biobanking studies (n = ≥5000). From the DCS biobank, we included a random cross-sectional sample for which a baseline plasma sample and yearly follow-up data were available (n = 750). For case-control analyses, this sample was supplemented with subjects selected for the inability to reach the glycemic target

Table 1. Baseline Clinical Characteristics of the Study Samples

Characteristic	DCS		Maastricht (n = 848)	CODAM (n = 134)	NEO (n = 664)
	Random Sample (n = 750)	Selected Sample (n = 245)			
Age, y	62.7 ± 10.2	63.5 ± 10.9	62.8 ± 7.6	61.1 ± 6.3	57.8 ± 5.4
Male sex	527 (57)	145 (59)	580 (68)	90 (67)	370 (58)
BMI, kg/m^2	30.7 ± 5.5	30.3 ± 5.4	29.9 ± 4.9	30.0 ± 4.3	33.0 ± 5.3
HbA1c, mmol/mol	46 (43–53)	53 (47–62)	50 (45–56)	50 (43–57)	48 (42–54)
HbA1c, %	6.4 (6.1–7.0)	7.0 (6.4–7.8)	6.7 (6.3–7.3)	6.7 (6.1–7.4)	6.2 (5.8–6.9)
HbA1c >53 mmol/mol	158 (21)	120 (49)	275 (32)	47 (35)	153 (23)
Diabetes duration, y	6.3 ± 4.7	7.6 ± 4.8	7.3 ± 6.8	3.2 ± 5.2	4.0 ± 5.1
Diabetes duration <1 y, n	36 (5)	8 (3)	134 (17)	77 (58)	277 (42)
Age at onset, y	56.9 ± 10.1	56.4 ± 10.6	55.6 ± 9.1	57.9 ± 7.1	52.0 ± 7.0
Statin use	524 (70)	162 (66)	627 (74)	31 (23)	344 (52)
Other lipid-lowering drug use	22 (3)	10 (4)	54 (6)	3 (2)	4 (1)
No medication	91 (12)	9 (4)	189 (22)	70 (52)	322 (48)
Metformin	275 (37)	40 (16)	264 (31)	7 (5)	153 (23)
Metformin + sulfonylurea	142 (19)	56 (23)	136 (16)	16 (12)	76 (11)
Sulfonylurea	50 (7)	19 (8)	20 (2)	28 (21)	17 (3)
Insulin	154 (21)	109 (45)	175 (21)	11 (8)	77 (12)
Other	38 (5)	12 (5)	63 (7)	2 (2)	19 (3)

Date are presented as mean ± SD, median (interquartile range), or n (%).

The DCS sample consisted of a random sample of 750 and a total sample in which 245 subjects with diabetic complications and/or unable to reach the clinical target of HbA1c where added to the random sample to increase power in case-control analyses.

[hemoglobin A1c (HbA1c) >53 mmol/mol] and/or experiencing diabetic complications (n = 245). For the prospective study, we used data from 596 patients from the random sample who were not taking insulin at the time of blood sampling for metabolomic data and for whom follow-up data were available. The follow-up time was 7 years (interquartile range, 6 to 7). HbA1c determination was performed using the turbidimetric inhibition immunoassay for hemolyzed whole EDTA blood samples (Cobas c501; Roche Diagnostics, Mannheim, Germany).

CODAM study

The CODAM study was started in 1999. The baseline measurements of the CODAM (n = 574) were obtained from 1999 to 2002 (12). The CODAM is a prospective, observational cohort. The general aim of the CODAM is to investigate the effects of glucose metabolism, lipids, lifestyle, and genetics on the development of T2D and its cardiovascular complications, with a focus on etiological relationships. For the present study, we included all subjects with T2D for whom a baseline plasma sample and HbA1c level were available (n = 134). For the prospective studies, we used data from 102 patients who were not using insulin at the time of blood sampling for metabolomic data and for whom follow-up data were available. The average follow-up time was 7 years (interquartile range, 6.9 to 7.1) (15). HbA1c was measured using ion-exchange HPLC.

The Maastricht study

The Maastricht study is an extensive phenotyping study that focuses on the etiology of T2D, its classic complications (*e.g.,* cardiovascular disease, nephropathy, neuropathy, and retinopathy), and its emerging comorbidities. The study represents a population-based cohort of 10,000 individuals that is enriched with participants with T2D participants. A detailed description of the study design has been reported by Schram *et al.* (11). For the present study, we included all subjects with T2D for whom a baseline plasma sample was available at the time of metabolite quantification (n = 848). One subject for whom detailed medication data were not available was excluded from the analyses involving medication data. HbA1c was measured using ion-exchange HPLC.

The NEO study

The NEO study was designed for extensive phenotyping to investigate pathways that lead to obesity-related diseases (13). The NEO study is a population-based, prospective cohort study that includes 6671 individuals aged 45 to 65 years, with an oversampling of individuals who are overweight or have obesity. For those with T2D at baseline, plasma samples were measured in the present study (n = 664). HbA1c was measured using HPLC boronate affinity chromatography.

The Rotterdam study

The Rotterdam study is a prospective population-based cohort study in Ommoord, a district of Rotterdam, Netherlands. The design of the Rotterdam study has been described in more detail previously (14). In brief, in 1989, all residents within the well-defined study area who were aged ≥55 years were invited to participate in the study. Of these residents, 78% (7983 of 10,275) agreed. The first examination was performed from 1990 to 1993. Subsequently, the follow-up examinations were conducted every 3 to 5 years. The present metabolomic study used plasma samples and baseline data collected during the third visit (1997 to 1999). The follow-up data were from the fourth visit (2002 to 2004). For the present study, we included 395 subjects with T2D who were not using insulin at the third study visit.

Glucose-lowering drug use

We defined six different treatment groups: (1) glucose-lowering drug treatment naive; (2) metformin monotherapy; (3) sulfonylurea monotherapy; (4) metformin and sulfonylurea combined; (5) insulin

therapy, with or without oral glucose-lowering drugs; and (6) use of oral glucose-lowering medication other than metformin and/or sulfonylurea. The other category consisted mainly of thiazolidinediones, with or without metformin and/or sulfonylurea. The clinical characteristics, medication use, and number of subjects per stratum per cohort are provided in Supplemental Tables 1–3.

Metabolomic measurements

Fasted EDTA plasma samples were analyzed in a single experimental setup on a high-throughput nuclear magnetic resonance platform as described previously (available at: www.nightingalehealth.com) (16, 17). In total, 162 metabolomic measures and/or derived composite scores (n = 12) were assessed, which represent a broad molecular signature of systemic metabolism. This included metabolites such amino acids, glycolytic intermediates, fatty acids and ketone bodies, and 141 other metabolomic measures such as mono-unsaturated and polyunsaturated fatty acids, glycerides, proteins, lipid concentrations, and compositions of 14 lipoprotein subclasses (Supplemental Table 6). A heatmap showing the correlation structure of the metabolomic measures in the DCS cohort is provided in Supplemental Fig. 1. These metabolomic measures were all in absolute molar concentration units.

Statistical analysis

Metabolomic measures in the different study samples were normalized using z-scaling after natural logarithmic transformation of the raw levels [ln(measure+1)], as suggested by the manufacturer and to facilitate cross-cohort comparisons. The HbA1c levels were logarithmically transformed before the analyses in each of the cohorts.

In each of the cohorts, linear and logistic per-measure regression models with adjustment for potential confounders (based on the reported data) were used to study continuous and binary outcomes, respectively. Only complete cases were used. Details are described for each of the main analyses. Bonferroni correction was applied to all analyses to account for multiple testing (162 tests; $\alpha \leq 3.1 \times 10^{-4}$). We chose to use the Bonferroni correction based on the number of metabolic measures tested but not to correct for the number of tests performed. Because of the high correlation between metabolites (~40 independent signals), this equates for the stratified analyses (n = 5) to an almost similar cutoff ($5 \times 40 = 200$ tests; $P \leq 2.5 \times 10^{-4}$ vs 3.1×10^{-4}). For the other endpoints (glycemic control and insulin initiation), for which we performed fewer tests, such a cutoff would have been too strict. Therefore, for uniformity and readability of our report, we chose to use one significance threshold throughout according to the number of metabolomic measures ($P \leq 3.1 \times 10^{-4}$). SPSS, version 23.0 (IBM Corp., Armonk, NY), and R, version 3.4.0 (R Foundation, Vienna, Austria), were used for data analysis. Random effect meta-analyses were used to combine the results of the different study samples using the R package meta (Meta, version 4.3-2; R Foundation) (18).

Association between metabolomic measures and HbA1c

The associations between metabolomic measures (main independent variables) and HbA1c levels (outcome) at the time of blood sampling were examined using linear regression models (n_{total} = 2641). Logistic regression was used to analyze the associations of metabolomic measures with insufficient glycemic control, defined as an HbA1c >53 mmol/mol (7%) at the time of the blood sampling. Two models were used. Model 1 included as covariates age, sex, statin use (yes vs no), and the use of other lipid-lowering medication (yes vs no). In model 2, we additionally adjusted for body mass index (BMI), the use of oral glucose-lowering medication (yes vs no), insulin use (yes vs no), and duration of diabetes at the time of blood sampling. Based on previous evidence, we examined the influence of the six different treatment regimens on the association between metabolomic measures and HbA1c in drug-stratified analyses. To examine differences between those without medication and other treatment groups, interaction analyses were performed (treatment group*metabolite). Sensitivity analyses were performed by excluding subjects with <1 year of diabetes and those only treated with a diet and in analyses stratified by sex.

Associations between glucose-lowering drug use and metabolomic measures

In a cross-sectional design, we applied linear regression analyses to examine the association between different types of glucose-lowering medication (main independent variable) and metabolomic measures (outcomes). Separate analyses for each treatment group with the treatment-naive group as the reference were used for each cohort separately. Analyses were restricted to the DCS, Maastricht study, and NEO cohorts because the numbers per stratum were too small in the CODAM cohort. Age, sex, statin use (yes vs no), and the use of other lipid-lowering medication were added as covariates (model 1). In model 2, we additionally adjusted for BMI, duration of diabetes, HbA1c, fasting glucose, and estimated glomerular filtration rate (eGFR) at the time of the blood sampling. eGFR was estimated using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation (19).

Association between metabolomic measures and initiation of insulin therapy

The metabolomic measures that were identified as cross-sectionally associated with HbA1c >53 mmol/mol in the previous analyses were included in the present analyses. The association between these baseline metabolomic measures (main independent variables) and the initiation of insulin therapy during the follow-up period (outcome) were examined with logistic regression in the prospective cohorts. For these analyses, we only included those who were not using insulin at the time of blood sampling (n = 698). The baseline values of age, sex, BMI, statin use, other lipid-lowering drug use (model 1) and diabetes duration, sulfonylurea use, metformin use, other diabetes medication use, HbA1c, and fasting glucose (model 2) were included as covariates. For replication in the Rotterdam study, we used a slightly different model that included age, sex, BMI, lipid-lowering medication use, oral glucose-lowering medication use, and fasting glucose, because not all covariates were available.

Because it is known that for various reasons, people who should use insulin to treat prolonged elevated HbA1c levels will not be using this drug, we performed sensitivity analyses in the largest prospective cohort, the DCS. Propensity scores for insulin use at baseline were calculated using graded boosting as implemented in the *gbm* package in R, version 2.1.3 (R Foundation) (20). Sex, age, BMI, diabetes duration, biobank year, HbA1c, fasting glucose, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, cholesterol ratio, triglycerides, and eGFR were used as variables.

Results

The cohort characteristics are listed in Table 1 and Supplemental Tables 1–5. Differences between cohorts in

diabetes duration and glucose-lowering medication use were accounted for using random effects meta-analyses. A schematic overview of the study and its main results is shown in Fig. 1.

Association between metabolomic measures and HbA1c

Using a linear regression model that include age, sex, and the use of statins or other lipid-lowering medication as covariates, we found substantial associations between metabolomic measures and HbA1c levels in all four cohorts. In the meta-analyses, 81 measures were significantly associated with the HbA1c levels after multiple testing correction (model 1; Supplemental Table 7). The most statistically significant association was observed with the Fischer ratio [BCAA/aromatic amino acids; β = 0.05 ± 0.00 (SE); P = 4.6×10^{-42}]. After further adjustment for BMI, glucose-lowering drug use, insulin use, and diabetes duration, 75 measures were statically significant associated (all $P \leq 3.1 \times 10^{-4}$ 67% overlap; model 2; Supplemental Table 7).

We next tested in a logistic regression model whether the metabolomic measures were also associated with the inability to achieve the glycemic target of an HbA1c ≤53 mmol/mol. A total of 26 measures (8 metabolites and 18 others) belonging to various metabolomic classes were significantly associated. The most statistically significant association was found for glutamine (OR, 0.66; 95% CI, 0.61 to 0.73; $P = 7.6 \times 10^{-19}$; Table 2; Supplemental Table 8). Most of these 26 were also significant in the same linear regression model (21 of 26 all $P \leq 3.1 \times 10^{-4}$) but not always in the extended model 2 (15 of 26).

In a sensitivity analysis, the exclusion of those with a <1-year duration of diabetes and those with diabetes only treated with a diet did not materially affect the results. This suggests that the observed associations were not driven by those with newly discovered or mild or screen-detected diabetes. We also did not observe major differences between men and women (data not shown).

We also tested whether the use of different glucose-lowering drugs affected the observed associations. Thus, we first evaluated whether the different treatment regimens were associated with the metabolomic measures in those patients on treatment compared with those who did not use any type of glucose-lowering drug. The results of the meta-analyses for the model adjusted for age, sex, BMI, statin use, and other lipid-lowering medications (5 metabolites; 21 others significant, all $P \leq 3.1 \times 10^{-4}$) are shown in Supplemental Table 9. With addition of diabetes duration, HbA1c, fasting glucose, and eGFR into the model, 31 measures (3 metabolites; 28 others all $P \leq 3.1 \times 10^{-4}$) remained significantly different in one or more of the treatment groups compared with those who did not use any type of glucose-lowering drug (Table 3; Supplemental Table 10). The metabolomic measures represent various categories, including amino acids, phospholipids, apolipoproteins (Apos), cholesterols, and various lipoprotein subclasses. The strongest association was observed for ApoA1 and metformin plus sulfonylurea dual therapy [$\beta = -0.148 \pm 0.026$ (SE); $P = 1.7 \times 10^{-8}$].

In the treatment group-stratified meta-analyses for the 26 measures identified in the logistic regression model for insufficient glycemic control, we found only modest evidence for an effect of medication on these associations (Supplemental Table 11). Only those in the small sulfonylurea monotherapy or "other" groups sometimes showed aberrant responses. However, in the interaction analyses of treatment group*metabolite, no statistically significant associations were found ($P \geq 8.5 \times 10^{-3}$ for all; data not shown). Altogether, these results imply that, in general, the major glucose-lowering drugs had little effect on the observed associations between metabolomic measures and HbA1c.

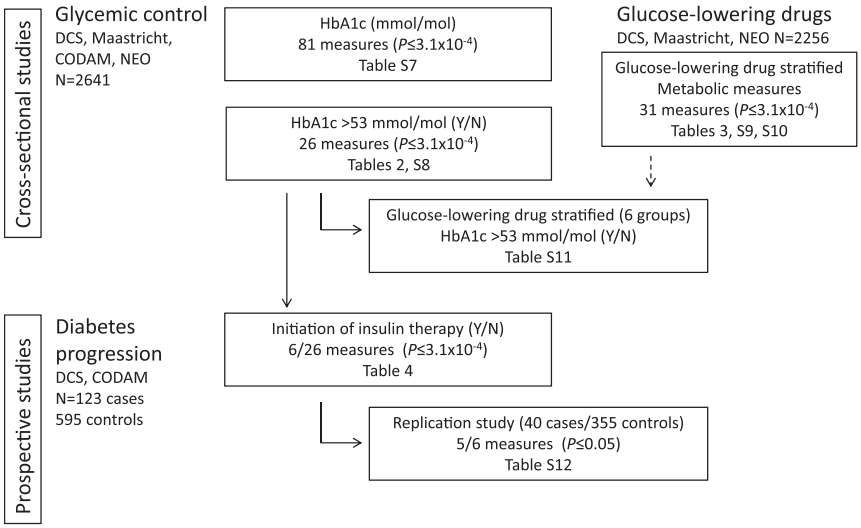

Figure 1. Schematic overview of the study design and main results. S, supplemental; Y/N, yes vs no.

Association between metabolomic measures and initiation of insulin therapy

Diabetes progression was defined as the initiation of insulin therapy during follow-up. Because the exact starting date of insulin therapy was not always known, we used logistic regression models for the prospective studies. However, Cox regression analysis in the DCS cohort showed highly similar results (data not shown). In a meta-analysis of the two cohorts with prospective data, we tested whether the 26 metabolomic measures we had identified were also

Table 2. Metabolomic Measures Significantly Associated With Insufficient Glycemic Control (HbA1c >53 mmol/mol)

	Model 1			Model 2		
Measure	**OR**	**95% CI**	***P* Value**	**OR**	**95% CI**	***P* Value**
Metabolite						
Gln	0.66	0.61–0.73	7.58×10^{-19}	0.66	0.57–0.76	1.51×10^{-8}
Ile	1.41	1.26–1.57	1.06×10^{-9}	1.40	1.22–1.60	1.63×10^{-6}
Leu	1.44	1.31–1.59	3.51×10^{-13}	1.46	1.23–1.74	1.32×10^{-5}
Val	1.46	1.33–1.60	2.74×10^{-15}	1.40	1.26–1.56	5.21×10^{-10}
BCAA	1.51	1.37–1.67	4.41×10^{-17}	1.48	1.32–1.65	3.84×10^{-12}
Fischer ratio	1.59	1.39–1.81	3.53×10^{-12}	1.49	1.25–1.79	1.61×10^{-5}
3-hydroxybutyrate	1.19	1.10–1.30	3.61×10^{-5}	1.11	0.99–1.24	6.16×10^{-2}
Lactate	1.26	1.14–1.40	1.20×10^{-5}	1.27	1.16–1.40	5.41×10^{-7}
Other metabolomic measures						
UnsatDeg	0.80	0.73–0.87	8.08×10^{-7}	0.81	0.74–0.90	5.51×10^{-5}
FAw3-FA	0.83	0.76–0.91	6.22×10^{-5}	0.90	0.81–0.99	3.68×10^{-2}
PUFA-FA	0.83	0.77–0.91	3.45×10^{-5}	0.82	0.73–0.93	2.18×10^{-3}
SFA-FA	1.23	1.10–1.36	2.08×10^{-4}	1.19	1.04–1.36	1.40×10^{-2}
LDL-TG	1.26	1.15–1.38	4.61×10^{-7}	1.33	1.20–1.48	3.05×10^{-8}
ApoA1	0.80	0.71–0.90	1.54×10^{-4}	0.96	0.84–1.09	4.82×10^{-1}
XS-VLDL-TG	1.26	1.13–1.40	2.47×10^{-5}	1.31	1.15–1.48	4.17×10^{-5}
IDL-TG	1.27	1.16–1.38	1.57×10^{-7}	1.32	1.19–1.46	6.47×10^{-8}
L-LDL-TG	1.25	1.14–1.38	4.46×10^{-6}	1.33	1.20–1.47	7.79×10^{-8}
M-LDL-TG	1.21	1.11 –1.33	2.33×10^{-5}	1.29	1.16–1.42	1.25×10^{-6}
S-LDL-TG	1.19	1.09–1.30	6.95×10^{-5}	1.26	1.14–1.40	3.31×10^{-6}
XL-HDL-FC	0.81	0.73–0.90	1.01×10^{-4}	0.89	0.80–0.99	4.00×10^{-2}
M-HDL-P	0.83	0.75–0.91	8.86×10^{-5}	0.96	0.83–1.12	6.36×10^{-1}
M-HDL-L	0.82	0.75–0.90	3.49×10^{-5}	0.96	0.82–1.12	5.81×10^{-1}
M-HDL-C	0.79	0.70–0.89	6.70×10^{-5}	0.90	0.77–1.06	2.17×10^{-1}
M-HDL-CE	0.78	0.70–0.88	5.05×10^{-5}	0.89	0.77–1.04)	1.57×10^{-1}
M-HDL-FC	0.80	0.72–0.90	2.19×10^{-4}	0.94	0.78–1.13	4.99×10^{-1}
S-HDL-TG	1.27	1.15–1.40	4.47×10^{-6}	1.26	1.12–1.42	1.17×10^{-4}

ORs and 95% CIs computed from fixed effect meta-analyses of the logistic regression analyses for insufficient glycemic control of DCS, Maastricht, CODAM, and NEO data.

Model 1 was adjusted for age, sex, statin use, and other lipid-lowering medication use. Model 2 was adjusted for age, sex, statin use, other lipid-lowering medication use, BMI, diabetes duration, oral hyperglycemic agent use, and insulin use.

Full data for all metabolomic measures are provided in Supplemental Table 8.

Abbreviations: FAw3-FA, ratio of ω-3 fatty acids to total fatty acids; IDL-TG, triglycerides in intermediate-density lipoprotein; L-LDL-TG, triglycerides in large LDL; LDL-TG, triglycerides in LDL; M-HDL-C, total cholesterol in medium HDL; M-HDL-CE, cholesterol esters in medium HDL; M-HDL-FC, free cholesterol in medium HDL; S-HDL-TG, triglycerides in small HDL; M-LDL-L, total lipids in medium LDL; M-LDL-TG, triglycerides in medium LDL; PUFA-FA, ratio of polyunsaturated fatty acids to total fatty acids; S-LDL-TG, triglycerides in small LDL; SFA-FA, ratio of saturated fatty acids to total fatty acids; UnsatDeg, estimated degree of unsaturation; XS-VLDL-TG, triglycerides in very small VLDL.

associated with the initiation of insulin therapy during the 7-year follow-up period (n = 698; 123 cases). Of the 26 metabolomic measures, 11 were significantly associated with insulin initiation (model 1; Table 4) compared with 15 of the remaining 136 metabolites (P for enrichment = 3.8×10^{-4}). The most statistically significant association was again with ApoA1 (OR, 0.52; 95% CI, 0.40 to 0.67; $P = 7.97 \times 10^{-7}$). Further adjustment for age, sex, BMI, statin use, other lipid-lowering drug use, diabetes duration, sulfonylurea use, metformin use, other diabetes medication use, HbA1c, and fasting glucose reduced the number of significant associations to six (all $P \leq 3.1 \times 10^{-4}$ model 2; Table 4). The most statistically significant association was with medium HDL cholesteryl ester (OR, 0.54; 95% CI, 0.42 to 0.71; $P = 4.5 \times 10^{-6}$). Independent replication (Rotterdam study, 40 cases and 355 controls; 5 years of follow-up) showed that five of these also showed directionally consistent evidence for a nominal association ($P \leq 0.05$) in the smaller replication study (Supplemental Table 12).

It is known that for various reasons, people who should use insulin because of prolonged elevated HbA1c levels are not using this drug; therefore, we performed some sensitivity analyses in the DCS study. We first calculated the propensity scores for using insulin at baseline based on the baseline characteristics of the participants either using or not using insulin. Adding these propensity scores to the regression models did not largely affect the results. Next, we reclassified as persons initiating insulin 11 who had elevated HbA1c levels on at least two of the yearly follow-up visits (HbA1c >64 mmol/mol). The results of this analysis did not materially affect our results nor did the

Table 3. Metabolomic Measures Significantly Associated With Glucose-Lowering Medication Use

Variable	Metformin (n = 732)	SU (n = 106)	Metf + SU (n = 410)	Insulin (n = 515)	Other (n = 132)
Metabolite					
Ala	0.241 ± 0.048[a]	−0.013 ± 0.050	0.142 ± 0.058	0.039 ± 0.046	0.073 ± 0.078
Val	0.182 ± 0.043[a]	−0.018 ± 0.042	0.193 ± 0.083	0.065 ± 0.043	−0.018 ± 0.034
BCAA	0.181 ± 0.047[a]	−0.006 ± 0.042	0.216 ± 0.085	0.049 ± 0.053	−0.012 ± 0.033
Other metabolomic measure					
SFA	−0.149 ± 0.099	0.023 ± 0.052	−0.051 ± 0.029	−0.165 ± 0.044[a]	−0.023 ± 0.047
HDL-D	−0.101 ± 0.042	−0.110 ± 0.048	−0.127 ± 0.026[a]	−0.174 ± 0.096	−0.040 ± 0.028
PC	−0.199 ± 0.065	−0.093 ± 0.048	−0.107 ± 0.028[a]	−0.425 ± 0.183	−0.035 ± 0.033
TC	−0.164 ± 0.065	−0.058 ± 0.048	−0.106 ± 0.028[a]	−0.332 ± 0.136	−0.011 ± 0.031
ApoA1	−0.154 ± 0.048	−0.157 ± 0.047	−0.148 ± 0.026[a]	−0.400 ± 0.161	−0.060 ± 0.028
HDL-C	−0.076 ± 0.042	−0.154 ± 0.048	−0.108 ± 0.026[a]	−0.233 ± 0.121	−0.050 ± 0.028
HDL2-C	−0.070 ± 0.043	−0.149 ± 0.049	−0.106 ± 0.026[a]	−0.184 ± 0.088	−0.051 ± 0.028
Serum-C	−0.160 ± 0.042[a]	−0.074 ± 0.041	−0.103 ± 0.024[a]	−0.347 ± 0.161	−0.029 ± 0.037
Free-C	−0.175 ± 0.051	−0.050 ± 0.043	−0.094 ± 0.024[a]	−0.287 ± 0.135	−0.022 ± 0.029
Est-C	−0.151 ± 0.041[a]	−0.081 ± 0.041	−0.104 ± 0.024[a]	−0.358 ± 0.168	−0.028 ± 0.038
IDL-L	−0.142 ± 0.039[a]	−0.043 ± 0.041	−0.073 ± 0.024	−0.242 ± 0.131	−0.003 ± 0.028
XL-HDL-P	−0.094 ± 0.043	−0.102 ± 0.051	−0.119 ± 0.027[a]	−0.143 ± 0.107	−0.048 ± 0.029
XL-HDL-L	−0.090 ± 0.043	−0.109 ± 0.049	−0.118 ± 0.026[a]	−0.146 ± 0.108	−0.046 ± 0.028
XL-HDL-PL	−0.095 ± 0.043	−0.073 ± 0.051	−0.116 ± 0.027[a]	−0.130 ± 0.100	−0.044 ± 0.030
XL-HDL-C	−0.071 ± 0.043	−0.132 ± 0.048	−0.108 ± 0.027[a]	−0.131 ± 0.102	−0.046 ± 0.028
XL-HDL-FC	−0.078 ± 0.044	−0.113 ± 0.050	−0.116 ± 0.027[a]	−0.142 ± 0.106	−0.048 ± 0.029
L-HDL-P	−0.084 ± 0.044	−0.113 ± 0.051	−0.122 ± 0.027[a]	−0.200 ± 0.120	−0.046 ± 0.030
L-HDL-L	−0.084 ± 0.044	−0.120 ± 0.049	−0.124 ± 0.026[a]	−0.210 ± 0.128	−0.044 ± 0.029
L-HDL-PL	−0.090 ± 0.044	−0.119 ± 0.049	−0.124 ± 0.026[a]	−0.228 ± 0.129	−0.046 ± 0.029
L-HDL-C	−0.070 ± 0.044	−0.113 ± 0.050	−0.117 ± 0.027[a]	−0.168 ± 0.116	−0.044 ± 0.029
L-HDL-CE	−0.067 ± 0.044	−0.113 ± 0.050	−0.116 ± 0.027[a]	−0.163 ± 0.114	−0.044 ± 0.029
L-HDL-FC	−0.078 ± 0.045	−0.110 ± 0.051	−0.118 ± 0.027[a]	−0.176 ± 0.115	−0.045 ± 0.030
L-HDL-TG	−0.169 ± 0.044[a]	−0.085 ± 0.054	−0.135 ± 0.028[a]	−0.346 ± 0.193	−0.021 ± 0.031
M-HDL-P	−0.123 ± 0.062	−0.163 ± 0.049	−0.106 ± 0.027[a]	−0.346 ± 0.122	−0.052 ± 0.034
M-HDL-L	−0.118 ± 0.061	−0.172 ± 0.050	−0.106 ± 0.026[a]	−0.342 ± 0.121	−0.049 ± 0.031
M-HDL-C	−0.096 ± 0.053	−0.184 ± 0.049[a]	−0.108 ± 0.027[a]	−0.314 ± 0.118	−0.048 ± 0.028
M-HDL-CE	−0.089 (0.051)	−0.184 ± 0.049[a]	−0.103 ± 0.027[a]	−0.297 ± 0.108	−0.048 ± 0.028
M-HDL-FC	−0.114 ± 0.057	−0.171 ± 0.049	−0.119 ± 0.027[a]	−0.356 ± 0.148	−0.048 ± 0.029

Data are presented as $\beta \pm$ SE from random effect meta-analyses of DCS, Maastricht, and NEO data of metabolomic measures against medication use with adjustment for age, sex, BMI, statin use, other lipid-lowering medication, diabetes duration, HbA1c, fasting glucose, and eGFR. Treatment-naive patients were used as a reference (n = 611) in separate analyses for each treatment group.

Abbreviations: Est-C, esterified cholesterol; Free-C, free cholesterol; HDL-C, HDL cholesterol; HDL2-C, HDL2 cholesterol; HDL-D, mean diameter for HDL particles; IDL-C, intermediate-density lipoprotein cholesterol; Metf, metformin; L-HDL-C, total cholesterol in large HDL; L-HDL-CE, cholesterol esters in large HDL; L-HDL-FC, free cholesterol in large HDL; L-HDL-P, concentration of large HDL particles; L-HDL-PL, phospholipids in large HDL; L-HDL-TG, triglycerides in large HDL; M-HDL-C, total cholesterol in medium HDL; M-HDL-CE, cholesterol esters in medium HDL; M-HDL-FC, free cholesterol in medium HDL; M-HDL-L, total lipids in medium HDL; M-HDL-P, concentration of medium HDL particles; PC, phosphatidylcholine; Serum-C, serum cholesterol; SFA, saturated fatty acids; SU, sulfonylurea; TC, total cholesterol; XL-HDL-C, total cholesterol in very large HDL; XL-HDL-FC, free cholesterol in very large HDL; XL-HDL-L, total lipids in very large HDL; XL-HDL-P, concentration of very large HDL particles; XL-HDL-PL, phospholipids in very large HDL.

[a]Bonferroni statistically significant associations ($P \leq 3.1 \times 10^{-4}$).

exclusion of these persons from our analysis (data not shown).

Discussion

The present study had several main findings (Fig. 1). First, in cross-sectional analyses, we showed that 26 measures were associated with insufficient glycemic control and were largely independent of the effects of glucose-lowering medications. Second, we identified 31 measures that differed between individuals treated with different glucose-lowering drugs. Finally, we showed in prospective analyses that 5 of the 26 measures associated with insufficient glycemic control were also associated with insulin initiation during follow-up.

Metabolomic measures and glycemic control

Increased levels of BCAAs, as observed in our study, were previously shown to be associated with insulin resistance and the risk of prevalent and incident diabetes (4, 21). We have now shown that this association extends to glycemic control in people with T2D. Glutamine, ranked first in our analyses, is known to be associated with insulin sensitivity and reduced diabetes risk, in line with our observed inverse correlation (6, 22, 23). Furthermore, we found positive associations with several markers of fatty acid composition

Table 4. Metabolomic Measures Significantly Associated With Insulin Initiation During Follow-Up

	Model 1			Model 2		
Measure	**OR**	**95% CI**	***P* Value**	**OR**	**95% CI**	***P* Value**
Metabolites						
Gln	0.86	0.70–1.07	1.73×10^{-1}	1.14	0.68–1.90	6.30×10^{-1}
Ile	1.58	1.22–2.04	5.71×10^{-4}	1.25	0.76–2.06	3.72×10^{-1}
Leu	1.54	1.23–1.93	1.77×10^{-4}	1.22	0.94–1.58	1.26×10^{-1}
Val	1.63	1.31–2.03	1.21×10^{-5}	1.20	0.75–1.94	4.50×10^{-1}
BCAA	1.72	1.37–2.17	3.86×10^{-6}	1.25	0.74–2.12	4.10×10^{-1}
Fischer ratio	1.79	1.42–2.26	1.15×10^{-6}	1.40	1.08–1.81	1.22×10^{-2}
3-hydroxybutyrate	1.03	0.84–1.26	7.59×10^{-1}	0.81	0.61–1.08	1.45×10^{-1}
Lactate	1.40	1.16–1.70	5.63×10^{-4}	1.06	0.66–1.69	8.10×10^{-1}
Other metabolomic measures						
UnsatDeg	0.73	0.58–0.92	7.04×10^{-3}	0.78	0.61–0.98	3.45×10^{-2}
FAw3-FA	0.74	0.52–1.05	9.39×10^{-2}	0.58	0.21–1.63	3.01×10^{-1}
PUFA-FA	0.84	0.56–1.27	4.17×10^{-1}	0.88	0.70–1.11	2.69×10^{-1}
SFA-FA	1.22	0.99–1.50	5.78×10^{-2}	1.10	0.88–1.37	4.15×10^{-1}
LDL-TG	1.01	0.59–1.70	9.82×10^{-1}	1.03	0.82–1.30	7.90×10^{-1}
ApoA1	0.52	0.40–0.67	7.97×10^{-7}	0.53[a]	0.39–0.70	1.31×10^{-5}
XS-VLDL-TG	1.18	0.73–1.90	5.02×10^{-1}	1.25	1.02–1.53	3.47×10^{-2}
IDL-TG	1.12	0.67–1.90	6.65×10^{-1}	1.21	0.97–1.50	8.95×10^{-2}
L-LDL-TG	1.01	0.60–1.70	9.58×10^{-1}	1.05	0.84–1.33	6.68×10^{-1}
M-LDL-TG	0.95	0.56–1.62	8.62×10^{-1}	0.98	0.78–1.23	8.53×10^{-1}
S-LDL-TG	1.06	0.62–1.81	8.32×10^{-1}	1.12	0.91–1.38	3.02×10^{-1}
XL-HDL-FC	0.59	0.46–0.75	1.86×10^{-5}	0.64	0.49–0.83	6.55×10^{-4}
M-HDL-P	0.56	0.44–0.72	5.06×10^{-6}	0.54[a]	0.41–0.72	1.52×10^{-5}
M-HDL-L	0.57	0.44–0.72	4.46×10^{-6}	0.55[a]	0.42–0.72	1.62×10^{-5}
M-HDL-C	0.56	0.44–0.70	1.24×10^{-6}	0.54[a]	0.41–0.70	4.67×10^{-6}
M-HDL-CE	0.56	0.44–0.71	1.30×10^{-6}	0.54[a]	0.42–0.71	4.46×10^{-6}
M-HDL-FC	0.55	0.43–0.70	2.62×10^{-6}	0.53	0.40–0.70	1.01×10^{-5}
S-HDL-TG	1.40	1.00–1.95	5.20×10^{-2}	1.37	1.10–1.69	4.21×10^{-3}

ORs and 95% CIs computed from fixed effect meta-analyses of the logistic regression analyses for insulin initiation in DCS and CODAM prospective data.

Model 1 was adjusted for age, sex, statin use, and other lipid-lowering medication use. Model 2 was adjusted for age, sex, statin use, other lipid-lowering medication use, BMI, diabetes duration, sulfonylurea use, metformin use, other diabetes medication use, HbA1c, and fasting glucose. Threshold for Bonferroni statistically significant associations ($P < 3.1 \times 10^{-4}$).

Abbreviations: FAw3-FA, ratio of ω-3 fatty acids to total fatty acids; IDL-TG, triglycerides in intermediate-density lipoprotein; L-LDL-TG, triglycerides in large LDL; LDL-TG, triglycerides in LDL; M-HDL-C, total cholesterol in medium HDL; M-HDL-CE, cholesterol esters in medium HDL; M-HDL-FC, free cholesterol in medium HDL; M-HDL-P, concentration of medium HDL particles; S-HDL-TG, triglycerides in small HDL; M-LDL-L, total lipids in medium LDL; M-LDL-TG, triglycerides in medium LDL; PUFA-FA, ratio of polyunsaturated fatty acids to total fatty acids; S-LDL-TG, triglycerides in small LDL; SFA-FA, ratio of saturated fatty acids to total fatty acids; UnsatDeg, estimated degree of unsaturation; XS-VLDL-TG, triglycerides in very small VLDL.

[a] $P < 0.05$ in the replication study (Supplemental Table 12).

and saturation and, respectively, positive and negative associations with concentrations of various very-low-density lipoprotein (VLDL), LDL, and HDL subclasses. Previous studies have shown that these measures are associated with various degrees of glucose tolerance, insulin resistance, and/or diabetes risk (24–27). In general, our data suggest that metabolomic measures that were previously shown to be associated with T2D risk are also associated with worse glycemic control.

Most of the associations with insufficient glycemic control were only marginally influenced by the different diabetes drugs in the stratified analysis. For example, in all treatment groups, insufficient glycemic control was positively associated with the Fischer ratio and most BCAAs. However, in the sulfonylurea group, no association or even an inverse association was found (Supplemental Fig. 2). For most of the fatty acids and lipoprotein subclasses, we noted similar findings in the sulfonylurea treatment group, with the associations less pronounced or the reverse of that observed for the other treatment groups. It seems that those in the "other" group, in general, showed stronger, but directionally consistent, associations. However, owing to the small numbers in both groups, the differences were not statistically significant and thus require further study. Metabolites such as glutamine and lactate showed much more similar associations in all treatment groups, suggesting a more generalized association of these metabolites with glycemic control. The differences in the associations observed in the various treatment groups were not explained by the differences in glycemic control, obesity, or diabetes duration. It is therefore reasonable to assume that they were related to differences in the working mechanism of these drugs targeting

either predominantly β-cell function or insulin action. Further studies are needed to investigate this in detail.

Diabetes treatment and metabolomic measures

To the best of our knowledge, we are the first to show the association of the different types of glucose-lowering drugs with the various metabolites and/or metabolomic measures in a large series of patients with T2D treated according to routine clinical care. Our results suggest that the observed differences were not strongly driven by differences in glycemic control or disease duration between groups. In general, it seemed that the direction and size of the effects were comparable between treatment groups, although not always reaching the formal levels of significance, which was likely due to the small numbers of patients in some subgroups. For example, it was previously shown that, among others, the phospholipid content of very large HDL was lowered by metformin treatment (8, 28). Our data suggest this was not specific for metformin but rather universal for most or all glucose-lowering drugs (Supplemental Fig. 3). Furthermore, individuals in most treatment groups, except for the "other" glucose-lowering drug group, had lower levels of HDL subclasses compared with those without glucose-lowering treatment (Supplemental Fig. 3). Because thiazolidinediones were included in this "other" group, this might relate to known HDL cholesterol increasing the effects of these drugs (29).

In addition to the generic effects of glucose-lowering drugs, we observed drug-specific associations. For instance, we show that compared to treatment naive patients, alanine levels are most strongly increased in metformin monotherapy or dual therapy with sulfonylurea groups. Confirming previous studies on metformin therapy (8, 30). BCAAs (Val, Leu, and Ile) and the Fischer ratio (BCAA/aromatic amino acid ratio) were increased in those treated with metformin. However, like alanine, these were not increased or were much less increased in those treated with sulfonylurea or other glucose-lowering drugs. This might be related to differences in the working mechanisms of these drugs.

Metabolomic measures and initiation of insulin therapy

For patients not able to achieve good glycemic control with oral glucose-lowering drugs, the initiation of insulin therapy is often the final treatment option. Patients with T2D who require insulin therapy have often been treated for years with oral glucose-lowering drugs without achieving sufficient glycemic control. This leads to an unwanted and prolonged exposure to high glucose levels and an increased risk of developing diabetes-related complications (2). Early indicators of treatment failure and rapid progression toward insulin therapy are thus urgently needed. We found that a subset of the metabolomic measures that were cross-sectionally associated with insufficient glycemic control were also associated with progression toward the requirement for insulin therapy during follow-up.

The BCAAs, although shown to be causally related to development of T2D (21), were not associated with the progression to insulin use. Also, other metabolites associated with insufficient glycemic control in our study were not associated with incident insulin use. Our data showed that high levels of ApoA1 and medium HDL subclasses were associated with an almost twofold reduced risk of incident insulin use. These findings have refined the results of previous studies that identified low HDL cholesterol as a risk factor for the initiation of insulin therapy (31) and the progression of glycemia in those with T2D (32). Insulin resistance impairs VLDL metabolism by (1) reducing the lipoprotein lipase-mediated generation of VLDL remnants and (2) simultaneously increasing the flux of adipose tissue derived fatty acids to the liver. Both processes lead to increased production of VLDL. The increased abundance of VLDL drives cholesteryl ester transfer protein–mediated transfer of cholesteryl ester from HDL to VLDL, leading to a reduction in HDL levels. Increased plasma VLDL and decreased HDL are characteristic of the so-called diabetic dyslipidemia [reviewed by Goldberg (33)]. Diabetic dyslipidemia represents a more advanced stage of insulin resistance and might thus identify those individuals more likely to progress toward insulin use. Alternatively, ApoA1 and HDL have also been suggested to modulate pancreatic β-cell function via incretin-like effects (34). Further detailed studies are needed to clarify this in detail.

The strengths of the present study were the use of large numbers of patients, incorporation of at least three independent cohorts in all main analyses, use of a targeted metabolomics platform already approved for clinical care, and use of stringent corrections for multiple hypothesis testing to reduce the chance of false-positive findings. One study limitation was the use of cross-sectional metabolomics data. Given our study design, we could not investigate the within-subject effects on the metabolomic measures after initiation of glucose-lowering treatment in treatment-naive individuals. Another limitation was the relatively small number of subjects in some of the treatment groups and the prospective studies, limiting the power to detect more modest associations. The use of logistic regression models for the prospective studies was a limitation; however, Cox regression analysis in the DCS cohort showed highly similar results. In addition, although we were able to show that several metabolomic measures were associated with incident insulin use, further studies using, for instance, lasso regression are warranted to find the best

combination of clinical and metabolomic predictors for the initiation of insulin therapy. However, this was beyond the scope of the present study. Finally, the metabolomics platform we used targets a relatively small and correlated number of metabolomic measures and is thus not representative of the whole metabolome. Because of the known correlation structure between the measures, the signals are not all independent but, rather, provide detailed information on the underlying biology. Further detailed metabolomic and lipidomic studies using specialized platforms to allow for more comprehensive and detailed analyses are needed to elucidate the underlying biology.

In conclusion, to the best of our knowledge, ours is the first study to show that blood metabolomic measures are associated with glycemic control. We also found that although the blood metabolome shows differences between patients taking different types of glucose-lowering medication, the glucose-lowering medication did not materially affect the associations with glycemic control. Finally, we found that the baseline levels of the metabolomic measures that were associated with insufficient glycemic control were also prospectively associated with the initiation of insulin therapy. Thus, metabolomic profiles might be useful for the identification of those at increased risk of treatment failure with noninsulin therapies.

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