A Predictive Metabolic Signature for the Transition from Gestational Diabetes to Type 2 Diabetes

Running title:

Metabolic signature for GDM to T2D transition

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Abstract

Gestational diabetes (GDM) affects 3-14% of pregnancies, 20-50% of which progress to T2D within 5 years. This study sought to develop a metabolomics signature to predict the transition from GDM to T2D. A prospective cohort of 1035 women with GDM pregnancy were enrolled at 6-9 weeks post-partum (baseline) and screened for T2D annually for 2 years. Of 1,010 without T2D at baseline, 113 progressed to T2D within 2 years. Another 17 developed T2D between 2-4 years. A nested case-control design utilized 122 incident cases matched to non-cases by age, pre-pregnancy BMI and race/ethnicity. We conducted metabolomics with baseline fasting plasma and identified 21 metabolites that significantly differed by incident T2D status. Machine learning optimization resulted in a decision tree modeling that predicted T2D incidence with a discriminative power of 83.0% in the training set and 76.9% in an independent testing set, being far superior to fasting plasma glucose alone. The ADA recommends T2D screening early post-partum via OGTT after GDM, a time consuming and inconvenient procedure. Our metabolomics signature predicted T2D incidence from a single fasting sample. This study represents the first metabolomics study of the transition from GDM to T2D validated in an independent testing set, facilitating early interventions.

SWIFT Study Clinical Trials.gov Registration Number: NCT01967030

Introduction

Currently, Gestational diabetes mellitus (GDM) occurs in 3-14% of pregnancies and 20-50% of women with GDM develop type 2 diabetes (T2D) within 5 years of the index pregnancy (1; 2). The American Diabetes Association (ADA) thus recommends T2D screening at 6-12 weeks postpartum and every 1 to 3 years thereafter via testing fasting plasma glucose (FPG), 2-hr 75 g oral glucose tolerance test (OGTT), or hemoglobin A1c for women in this high risk population (3). However, screening of women post-GDM pregnancy remains sub-optimal, with very low compliance rates of 16-19% (4; 5), although integrated health care systems report 60% screening (2). Reasons for low rates include logistical difficulties of administering an oral glucose tolerance test (OGTT), fear of receiving a diagnosis of diabetes (6) and failure to attend the post-partum follow-up exam (7). Furthermore, many women with a previous GDM pregnancy hold a faulty low risk perception of T2D incidence (8; 9). A metabolic risk score that can quantify risk, for prediction of the transition from GDM to T2D with a single non-fasting test, would thus be beneficial, but is currently unavailable. Although several risk scores have been developed for T2D (10; 11), none of them consider a history of GDM diagnosis. Thus, prediction of T2D in women with a previous GDM pregnancy is critical for individual risk stratification and early prevention following delivery.

Herein, we have used a metabolomics approach that implements advanced machine learning methods as an excellent tool to identify early diagnostic biomarkers that have the best predictive abilities for complex pathologies such as diabetes, a heterogeneous disorder of glucose metabolism that can have diverse root cause across various racial and ethnic subgroups (12). We measured numerous metabolites in stored frozen fasting plasma samples drawn at 6-9 weeks post-partum under standardized research protocols from women with recent GDM without diabetes via the 2-hr

75g OGTT and in whom annual follow-up screening (2-hr 75 g OGTT) was conducted to identify new onset of T2D within two years.

Previous metabolomic investigations of T2D in the general population have revealed significant differences between diabetic patients and normal glucose tolerant (NGT) controls (13-22), although the majority of these were cross-sectional studies of T2D prevalence. Recently a study performed lipodomic analysis and evaluated risk of T2D among women with previous GDM of northern European ancestry (23). In this study, clinical variables combined with lipid species predicted 21 cases of T2D during 8.5 years of follow-up with over 80% accuracy. However, this signature has not been independently validated, or tested among other ethnicities. Thus, there is an unmet need to accurately predict T2D after GDM pregnancy with a more convenient and accurate method. This study represents the first metabolomics study of the transition from GDM to T2D and offers a quantitative measure of risk, as well as insight into etiology of the transition.

Research Design and Methods

Study Design

The Study of Women, Infant Feeding, and Type 2 diabetes mellitus after GDM Pregnancy (SWIFT) is prospective cohort study that enrolled 1035 racially and ethnically diverse women (aged 20-45 years) who were diagnosed with GDM via a 3-hr 100 g OGTT based on Carpenter and Coustan criteria, had no prior history of diabetes or other serious health conditions, received prenatal care and delivered singleton pregnancies of 35 weeks gestation or longer at a Kaiser Permanente Northern California (KPNC) hospital during 2008-2011. Details of the study recruitment, selection criteria, methodologies and baseline characteristics of the cohort (75% minority women; Asian, Hispanic, and Black, and 25% low-income) have been described previously (24; 25). The SWIFT

Study participants provided written consent to attend three in-person study visits at baseline (6-9 weeks post-partum), 1 year and 2 years post-partum that included 2-hr 75 g OGTT and assessments of lactation, intensity and duration, socio-demographics, medical and reproductive history, lifestyle behaviors and anthropometry (24). At each study visit, trained research staff collected and processed plasma samples at the fasting and 2-hr time points during the 75 g OGTT and completed assessments. These plasma samples were analyzed within several weeks for glucose, insulin and subsequently for selected lipids and lipoproteins, as previously described (25; 26). The study design and all procedures were approved by the KPNC Institutional Review Board for the protection of human subjects. Of 1,010 women without T2D at baseline, 959 (95%) had follow-up assessments for T2D status within two years after baseline via annual study OGTTs and electronic medical records to capture diagnoses of diabetes from KPNC clinical laboratory tests within and beyond the 2 years post-baseline (27). T2D diagnosis was based on ADA criteria (24).

Design of Experiment

Of the 130 incident T2D cases, 113 developed within 2 years post-baseline (27), and another 17 beyond 2 years as of December 2014. Using a nested case-control study design within the prospective cohort, 122 cases (105 within 2 years, and 17 beyond 2 years post-baseline) were matched to non-T2D controls in a 1:1 ratio based on age, pre-pregnancy BMI and race/ethnicity. Age, pre-pregnancy BMI, and ethnicity/race distribution in these excluded cases were not significantly different from cases included in the analysis. The 122 incident T2D cases were split in a 2:1 ratio for the training and testing sets. Importantly, the training set cases were all time-matched to incidence within 2 years, and were used to develop a metabolic risk signature. Subsequently, the testing set, comprising 28 cases within 2 years as well as 14 cases beyond 2 years, was used to independently ensure generalizability of the model.

Metabolite Assay Development

To assay all metabolites of interest, a total of 182 metabolites were subpanelled into 4 major methods and evaluated in fasting plasma samples collected at 6-9 weeks post-partum. The subpanel of 13 free fatty acids and 4 amino acids were selected based on a literature review of over a dozen of T2D metabolomics studies (13-22; 28; 29). These metabolites were chosen on the basis of consistency in trend direction and significance in a minimum of two studies. Both free fatty acid and amino acid subpanel assays were developed in-house as described below in the following relevant sections. In addition, a total of 163 metabolites were assayed using the p150 Absolute*IDQ*™ plate technology according to the manufacturer's instructions (Biocrates Life Sciences AG, Austria). All assays were performed by the Analytical Facility for Bioactive Molecules (The Hospital for Sick Children, Toronto, Canada). Beta-hydroxybutyrate (BHB 700190; Cayman Chemicals, USA) was assayed by ELISA while Fasting (FPG) and 2-hour OGTT post-load glucose (2hPG) were assayed as previously described (25). Only metabolites with a coefficient of variation (CV) of <20% for each batch were accepted for the multiplex methods, although the majority had CV of <15%. In addition, values were only accepted if the read concentration was within the dynamic range of the assay.

Amino Acid Analysis

For amino acid analyses, aliquots (10 µL) of plasma samples and standard mix samples (0.05-50 µg/mL Leu and Ile, 0.005-5 µg/mL AAA and PAG) were spiked with the internal standard mixture (5 µg/mL Leu-d10 and Glu-d3, 0.5 µg/mL PAG-d5 in H2O + 0.1% FA) and extracted by protein precipitation using 600 µL methanol. Samples were then derivatized with 100 µL 3N HCL in n-butanol, evaporated, and reconstituted in 500 µL of the LC/MS/MS mobile phase. LC-MS/MS analysis was performed on an Agilent 1290 HPLC with a Q-Trap 5500 mass spectrometer (AB

Sciex). Chromatography was performed isocratically on a Kinetex HILIC column (2.6 μm 100 Å, 50 x 4.6 mm) (Phenomenex) at a flow rate of 500 μL/min using 5 mM ammonium formate (pH 3.2) in 10/90 water/acetonitrile as the mobile phase. Data was acquired by scheduled MRM.

Free Fatty Acids Analysis

For selected fatty acids, aliquots (20 μL) of plasma samples and standard mix samples [(palmitic (C16:0), palmitoleic (C16:1 n-7), cis-7-hexadecenoic (C16:1 n-9), stearic (C18:0), oleic (C18:1 n-9), vaccenic (C18:1 n-7), linoleic (C18:2), α-linolenic (C18:3), arachidic (C20:0), eicosenoic (C20:1 n-7), arachidonic (C20:4), eicosapentaenoic (EPA; C20:5), docosapentaenoic (DPA; C22:5), and docosahexaenoic (DHA; C22:6) acids)] were spiked with internal standards [(myristic acid-d3 (C14:0-d3), palmitoleic acid-d14 (C16:1-d14), heptadecanoic acid (C17:0) and eicosanoic acid-d3 (C20:0-d3))]. Samples were then acidified with 1 M HCl, and extracted twice with 1 mL of hexane. The combined hexane phases were taken to dryness and derivatized with equal amounts of 1% pentafluorobenzyl bromide and 1% diisopropylamine, evaporated, and reconstituted in 200 μL of hexane. The samples were then injected on the GC-MS system. Excellent separation on the chromatograph was observed for every fatty acid, except for oleate and vaccenate. These two were thus combined to give a total concentration for C18:1

Statistical analysis

Testing and training set characteristics at baseline were compared using chi-squared statistics for categorical variables (race, education, perinatal characteristics, medication use) and by comparison of means for continuous variables using analysis of variance (fasting plasma lipids and glucose, age, BMI). A two-tailed independent t-test was computed to determine significant differences between non-T2D and incident T2D in the baseline metabolite concentrations, with alpha value set at $p<0.05$

using SPSS Statistics version 20 (SPSS Inc. IBM: USA) and then *p*-values corrected for multiple comparisons with the Benjamini-Hochberg method using RStudio software version 0.99.486 (Boston, MA, USA). Predictive modelling was performed using WEKA (University of Waikato, New Zealand). The best model was selected as the one with the highest score in the summation of the discriminative power from the receiver operating curves (ROC) and the F-score (30), a measure that places greater weight on detecting future cases. The J48 machine learner was optimized to develop a broad classifier by setting the confidence threshold to 0.5 and the minimum object in the leaf node to 14. The Naïve Bayes classifier was used as the default parameter setting in the WEKA software. Sensitivity, specificity and precision were further calculated from the classification plot for both the training and testing set.

P*earson's correlation* coefficients were calculated to analyze the relationship between significant metabolites and baseline clinically-relevant parameters baseline BMI, FPG, 2-hPG, fasting insulin, and HOMA-IR) using SAS for Windows (9.1.3, SAS Institute Inc., Cary, NC, USA).

Results

Baseline sociodemographic and clinical characteristics of training and testing sets are summarized in Table 1. While the mean age of women in the training set was significantly younger ($p<0.05$) compared to testing set, no statistically significant differences in any other baseline or prenatal clinical characteristics were found. The race/ethnicity distribution in both training and testing sets were similar. There was no statistically significant difference in either pre-pregnancy or baseline (6-9 weeks post-partum) BMI, total caloric intake or physical activity. A greater proportion of T2D incident cases had a family history of T2D in the testing set compared to the training set. At

baseline, there were statistically significant higher mean FPG, 2-hPG, hPG, fasting insulin and a higher proportion treated with insulin or oral diabetes medications during pregnancy among incident T2D compared to non-T2D ($p<0.05$) in both sets. Mean HOMA-IR was higher for T2D versus non-T2D ($p<0.05$) only in the training set.

A total of 110 metabolites passed all quality control criteria as described above. In the training set, a two-tailed independent *t*-test was carried out, with 21 metabolites found to significantly differ between T2D and non-T2D (Table 2). The metabolites 2-aminoadipic acid ($p<0.008$), Ile ($p<0.008$), Leu ($p<0.006$), Thr ($p<0.01$), Trp ($p<0.01$), Tyr ($p<0.0007$), Val ($p<0.001$), xLeu ($p<0.0008$), Hexose ($p<0.000001$) and AC3 ($p<0.04$) levels were significantly elevated in incident T2D compared to non-T2D. In contrast, metabolites Gly ($p<0.03$), SM (OH) C16:1 ($p<0.03$), SM (OH) C22:2 ($p<0.03$), SM C18:0 ($p<0.02$), SM C18:1 ($p<0.004$), SM C20:2 ($p<0.001$), SM C24:1 ($p<0.01$), PC ae C40:5 ($p<0.04$), PC ae C42:5 ($p<0.02$), PC ae C44:5($p<0.04$), AC10 ($p<0.04$) and free fatty acid palmitoleic acid (C16:1 n9) ($p<0.03$) were decreased in incident T2D compared to non-T2D. Furthermore, Tyr, Val, xLeu, hexoses and SM C20:2 remained statistically significant after *Benjamini-Hochberg correction* for multiple comparisons (Table 2).

To identify a set of metabolites with accurate prediction of future T2D we selected a rigorous method of splitting data into training (model building) and testing (model verification) over methods such as cross validation and holdout. Several methods of attribute selection were explored. First, attributes were ranked by predictive capacity and then trained and tested in a Naïve Bayes model. While this initial model worked well in a 10-fold cross-validation it performed poorly in the testing set, indicating that this method of attribute selection contained dataset specific biases (data not shown). Next, the J48 decision tree method using random sampling of attributes to build trees

and then select and prune the trees to identify the best preforming attributes (the metabolite model) was used to create the model. We optimized the J48 model by increasing the confidence threshold to 0.5 and the minimum number of subjects to 14. These settings ensured a broad classifier model not prone to over fitting.

The resulting metabolite model had a high summation of AUC and F-score in the training set (Fig 2A), relying on only a few metabolites: PC ae C40:5, hexoses, BCAA (Val, Leu, Ile), and SM (OH) C14:1. Baseline (6-9 weeks post-partum) FPG alone predicted T2D incidence in the training set, with an AUC of 0.724 (95% CI, 0.645-0.803, $p<0.0001$), sensitivity 60.0%, specificity 75.0%, F score 0.649 and total score 1.373. In contrast, the metabolite model resulted in an AUC of 0.830 (95% CI, 0.765-0.894, $p<0.000001$), with sensitivity 86.3%, specificity 69%, F score 0.793 and total score 1.623. We next applied the metabolite model and the FPG model against the testing data set and assessed relative performance using ROC curves (Fig 2B). The FPG model was worse at predicting T2D, with AUC 0.706 (95% CI, 0.569-0.816, $p<0.01$), sensitivity 57.0%, specificity 66.7%, F score 0.6 and total score 1.306. In contrast, the metabolite model performed well with an AUC 0.769 (95% CI, 0.667-0.871, $p<0.001$), sensitivity 73.8%, specificity 69%, F score 0.721 and total score 1.49 (Table 3). The metabolite model also outperformed the use of 2hPG in both the training set (AUC 0.726, F score 0.6309, total score 1.357) and testing set (AUC 0.661, F-score 0.615, total score 1.276).

Using FPG and the 2hPG we could build a model using J48 decision tree method (the glucose model). The glucose model had greater sensitivity (Se) but worse precision (P) and specificity (Sp) compared to the metabolite model (glucose model P=0.627, Se=0.881, Sp=0.476; metabolite model: P=0.705, Se=0.738, Sp=0.690). To determine if combining the glucose model and metabolite model

(the combined model) could improve prediction we built an optimized Naïve Bayes classifier model combining the four metabolites species and glucose data (FPG and 2hPG). The combined model showed worse prediction compared to metabolites alone (P=0.697, Se=0.548, Sp=0.762). Of the three models, the metabolite only model outperformed the latter two models with the highest AUC and F score (Table 3). The predictions from the three models (metabolite, glucose and combined metabolite-glucose) were directly compared in a Venn diagram to determine the similarities and differences between the models (Fig 3).

From the comparisons of the three models (Fig 3) the combined model showed improvement in capturing all 6 future T2D cases solely predicted by the glucose model and missed by the metabolite model. The glucose model could only capture 11 of 16 future T2D cases predicted by the metabolites model. The combined model fared worse in prediction of controls with 8 unique false negatives (predicted as diabetic; Fig 3).

Pearson correlation coefficients were calculated between the 22 metabolites that significantly differ between incident T2D and non-T2D in the training set, metabolite selected by machine learning and 5 baseline clinical parameters that significantly differed between incident T2D and non-T2D in both training and testing sets (BMI, fasting glucose, 2-hour post-load glucose, fasting insulin and HOMA-IR). SM C24:1 most significantly and negatively correlated with BMI ($p<0.0005$, r= -0.277). The correlations of 2-AAA, Ile, AC3, hexoses and SM C20:2 were most significant with fasting glucose level ($p<0.0005$, r= 0.283, 0.278, 0.306, 0.826, and -0.284, respectively). After 2-hr post-load, total hexoses were most significantly correlated with glucose levels (p<0.005, r=0.211) as expected. All other metabolites, with the exception of palmitoleic acid, significantly correlated with both fasting insulin and HOMA-IR (Table 4). Interestingly, among all 22 significant

metabolites, glycine and hexoses were the only metabolites to correlate significantly to all 5 clinical parameters; BMI (r = -0.151, 0.160), fasting glucose (r = -0.192, 0.826), 2-hour post-load glucose (r = -0.173, 0.211), fasting insulin (r = -0.279, 0.311) and HOMA-IR (r = -0.281, 0.429). SM (OH) C14:1 correlated negatively with BMI, FPG, 2hPG, fasting insulin and HOMA-IR like other SMs investigated in this study.

Discussion

GDM represents one of the strongest risk factors for the development of T2D, and identifies young women of whom 20-50% may develop T2D within 5 years after delivery (1). Metzger et al. reported that greater severity of hyperglycemia during pregnancy predicted T2D conversion within 6 months post-partum as opposed to 5 years, and that higher pre-pregnancy BMI increased the risk of T2D within 5 years post-partum (31). The Diabetes Prevention Research Group reported a greatly reduced risk of T2D progression among women with a history of GDM by either a lifestyle modification or metformin treatment, with T2D incidence of 10-15% within 10 years compared to 50% in the standard care group (32). Nevertheless many women with GDM hold a false perception of low risk status for future diabetes (8; 9). Thus, diabetes screening is suboptimal during the post-partum period because of the time-consuming glucose tolerance testing and required fasting period.

Herein, we explored a combination of several significantly altered metabolites for prediction of incident T2D compared with clinical parameters, the FPG and 2hPG among women matched on age, race/ethnicity and BMI. Our metabolite model predicts T2D above and beyond the risk contributed by obesity. Several metabolites were statistically significant predictors of incident T2D and they were previously associated with T2D in cross-sectional metabolomics studies, suggesting that GDM women at risk of progressing to T2D present a more T2D-like metabolte profile within

the very short time frame of 2 months post-partum compared to women who will remain non-diabetic. Women who developed T2D were also more likely to have been treated with insulin or oral medication during pregnancy, underscoring the predictive value of the severity of glucose intolerance during pregnancy.

Comparison of the three T2D predictive models identified the metabolite model as the most balanced for type-I (false positive) and type-II (false negative) error over the glucose model. A combined model of metabolites and glucose could improve capture of future T2D over glucose alone, but with higher false positive prediction rates. This increased type-I error suggests a conflict between the predictions arising from the metabolite or glucose models. Alternatively, these false positive predictions of future diabetes may represent detection of individuals that will develop diabetes beyond the two-year window of our current study.

Several amino acids (2-AAA, Ile, Leu, Thr, Trp, Tyr, Val) were increased in incident T2D subjects, except for glycine, which was significantly decreased and is a known predictor of T2D (19). The metabolite 2-AAA has been reported to be increased up to 12 years before T2D onset (28). Interestingly, 2-AAA was elevated in women with incident T2D after a previous GDM pregnancy, and positively correlated with insulin resistance. In our study, we also observed increase in levels of 2-AAA in incident T2D women. However in a study by Fiehn et al, where levels of 2-AAA were assessed in a cross-sectional study of African American women with T2D, no statistical significance was observed (15). Mechanistically, in murine models treated with 2-AAA decreased FPG and enhanced glucose-stimulated insulin secretion in beta cell models were observed (28). It is still to be determined if a similar response exists in humans.

BCAA levels correlate with insulin resistance in obese subjects (34). Catabolism of BCAAs plays an important role in T2D and Impaired Fasting Glucose (35). Clinical trials have also demonstrated that BCAAs such as leucine, isoleucine and valine are increased up to 7 years before T2D onset (18). In this study, BCAAs were elevated at 6-9 weeks postpartum among women at highest risk of subsequent progression to T2D, indicating that this metabolic profile precedes the onset of disease rather than being a consequence of T2D.

In our cohort, we observed higher levels of the hexoses (all 6 carbon sugars such as glucose, fructose and mannose) for incident T2D, consistent with others (18). Interestingly, in a T2D metabolomics study, Fiehn et al characterized carbohydrates, and found fructose levels to be significantly elevated in obese women with T2D (15). Unlike glucose, fructose stimulates hepatic lipogenesis which may result to hepatic insulin resistance, a key feature of T2D (36).

We also observed an overall reduction of sphingomyelin species in incident T2D compared to non-T2D. Wang et al. confirmed a decrease in SMC20:2, SM C16:0, SM C16:1, among other SM species (19), and Floegel et al observed a decrease in SM C16:1 and an inverse association with insulin secretion (21). In these nested-case control studies, the decreases were found up to 7 years before T2D incidence. The metabolic breakdown of SM results in ceramides, which is a known to induce beta cell apoptosis (37; 38). Further research is required to determine whether altered concentrations of ceramides mechanistically contribute to T2D and specifically to levels of SM C20:2, the sphingomyelin species most significant in this cohort.

Anderson et al investigated the lipidome of postpartum women who were normal, and hyperglycemic (non-GDM) or GDM. They observed that phosphatidylcholine, lysophosphatidylcholine, acylcarnitines and free fatty acids had the strongest correlations (39). Lappas et al applied lipidomics analysis of plasma collected at 12 weeks post-partum in 104 women with a GDM pregnancy who had normal postpartum glucose tolerance (NGT) and later evaluated T2D again at 8-10 years after delivery (23). A model including age, BMI, pregnancy FPG, postnatal FPG, triacylglycerol and total cholesterol and 3 metabolites (CE 20:4, PE(P-36:2) and PS 38:4). In our study, palmitoleic acid, AC3 and AC10 were significantly altered with incident T2D. Palmitoleic acid levels were positively related to T2D among older adults (40), and AC3 is known to be integral in the pathway of BCAA catabolism (34). In previous studies, AC10 has been associated with a graded increase among NGT, IGT and T2D individuals, but others found no significant difference in AC10 for T2D compared to control women (14; 41). In contrast, our study revealed a decrease in AC10 levels.

Prediction revealed two novel metabolites, PC ae C40:5 and SM (OH) C14:1 as predictive of incident T2D. Interestingly, PC ae C40:5 was not only significantly decreased in incident T2D but also negatively correlated with BMI, fasting insulin and HOMA-IR. Importantly, machine learning selected metabolite SM (OH) C14:1, a metabolite not associated with T2D incidence. This is because in predictive modeling, as opposed to traditional exploratory research, association is not a requirement for variable inclusion (42). Interestingly, similar to other SM, SM (OH) C14:1 correlated negatively with BMI, FPG and 2hPG, which may partially explain why the combined model did not outperform the metabolite-only model.

Presently, the ADA recommends T2D screening via fasting glucose or the 2-hr 75 g OGTT at 6-12 weeks post-partum and thereafter every 1-3 years for women with a prior GDM diagnosis, and more frequent testing if screening results fall within the pre-diabetes ranges. Our metabolomics signature holds the potential to replace the requirement for frequent OGTTs, Surpassing both the issue of lost follow-up and low screening rates with a single fasting measurement. In addition, this signature was comparable and outperformed using the 2-hour post-load plasma glucose after the OGTT in predicting future T2D incidence within 2 years. Furthermore, this signature presents valuable insight into etiology of the transition to T2D in women with previous GDM.

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The authors declare that there is *no conflict of interest*. A Allalou, EP Gunderson and MB Wheeler are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Abbreviations

2-AAA	2-aminoadipic acid
2hPG	2 hour post-load plasma glucose after 75 gram OGTT
AC	Acylcarnitines
ADA	American Diabetes Association
Arg	Arginine
AUC	Area under the curve
BCAA	Branched chain amino acids
BMI	Body mass index
CV	Co-efficient variation
DT	J48 decision tree
FFA	Free fatty acids
FPG	Fasting plasma glucose
FPIC	Female plasma internal standard
GDM	Gestational diabetes mellitus
Gln	Glutamine
Gly	Glycine

HDL	High density lipoprotein
His	Histidine
Ile	Isoleucine
Leu	Leucine
LR	Logistic regression
LLOQ	Lower limit of quantification
LOD	Limit of detection
LPC	lysophosphatidylcholine
Met	Methionine
NB	Naïve Bayes
NGT	Normal glucose tolerant
non-T2D	Did not develop type 2 diabetes
OGTT	Oral glucose tolerance test
Orn	Ornithine
PAG	Phenyl acetyl glutamine
PC	Phosphatidylcholine
PG	Plasma glucose
Phe	Phenylalanine
Pro	Proline
QNT	Quantitative
ROC	Receiver operating curve
Se	Sensitivity
Ser	Serine
SM	Sphingolipids
Sp	Specificity
SQ	Semi-quantitative
SWIFT	Study of Women, Infant Feeding, and Type 2 diabetes mellitus after Gestational Diabetes
T2D	Type 2 diabetes
Thr	Threonine
Try	Tryptophan
Try	Tyrosine
Val	Valine
Xleu	xleucine

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Titles and Legends

Title **Figure 1: Study design and metabolic assay work flow**

***Legend* Figure 1: A**) Study design of the SWIFT prospective cohort, a total of 1035 women diagnosed with GDM were enrolled at 6-9 weeks post-partum (baseline) and screened via 2-hr 75 g OGTTs. At baseline (V1), 21 women with T2D and 4 ineligibles were excluded from the follow up. The study followed 1,010 participants without diabetes who were re-screened annually via OGTTs with retention rates of 85% and 83% for 1 and 2 years, respectively (95% retention overall up to 2 years). Prospective cohort sample sizes for non-T2D and incident T2D shown: 59 developed T2D at 1 years and 54 developed T2D at 2 years, and another 17 women developed T2D beyond 2 to 4 years post-baseline. **B)** Work flow of metabolomics assay. A total of 182 metabolites were assayed in plasma from V1 (baseline) using LC-MS/MS, GC-MS and ELISA. For detailed methodology, please refer to the supplemental methods.

Title **Figure 2: Decision tree and ROC for the prediction of Incident T2D.**

***Legend* Figure 2: Decision tree and ROC for the prediction of T2D. A)** Decision tree by J48 based on the combined AUC and F-score of all algorithms. The grey boxes indicate the metabolite chosen for the node, while the clear numbered boxes indicate the concentration threshold in µM for PC ae C40:5, BCAA and SM (OH) C14:1 and mM for hexoses. **B)** ROC of the J48 algorithm on the training and testing set, performing with discriminative power 0.830 (p<0.000001) and 0.769 (p<0.0001), respectively, which is greater than FPG alone 0.724 (p<0.0001) and 0.706 ($p<0.01$), as well as 2hPG alone 0.726 (p<0.000001) and 0.661 (p<0.05), respectively. Data presented in AUC.

Title Figure 3: Venn diagrams and contingency tables comparison of model predictions of future diabetes.

Legend Fig 3: Venn diagrams and contingency tables comparison of model predictions of future diabetes. A) Venn diagrams of correct and incorrect predictions of the testing data set for all patients, only incident T2D and only non-T2D (Non) patients are shown. Intersection of correct predictions (green) and incorrect (red) indicates that one or more models had identical prediction of a patient and the other model/s did not. While the correct and incorrect patient predictions appear similar across all three models (left), the glucose and combined models have worse performance for prediction of future diabetes (middle). The combined model has worse prediction of controls (right). **B)** Contingency tables of the three different models against the testing data set. Columns are known group labels and rows are predicted group labels. The metabolite model (left) shows the higher precision and specificity compared to the glucose model. The combined model (right) has overall poorer sensitivity and specificity compared to both the metabolite and glucose models alone.

Characteristics	Training Set		Testing Set	
	Non-T2D (n=80)	**Incident T2D (n= 80)**	**Non-T2D (n=42)**	**Incident T2D (n= 42)**
Sociodemographic/Clinical				
Age, years	33.1 (4.5)	33.3 (5.2)	**35.1 (5.5)†**	**35.4 (5.5)†**
Race/Ethnicity, n				
Non-Hispanic White	13 (16)	12 (15)	8 (19)	9 (21)
Asian, (East, South, Southeast)	26 (33)	26 (33)	13 (31)	10 (24)
Non-Hispanic Black	10 (12)	10 (12)	2 (5)	5 (12)
Hispanic	31 (39)	31 (39)	17 (41)	17 (41)
Other	0 (0)	1 (1)	2 (5)	1 (2)
Parity, n				
Primiparous (1 birth)	31 (39)	26 (33)	13 (31)	16 (38)
Biparous (2 births)	27 (34)	29 (36)	14 (33)	16 (38)
Multiparous (>2 births)	22 (27)	25 (31)	15 (36)	10 (24)
GDM prenatal treatment, n		Chi-sq *		Chi-sq *
Diet Only	50 (63)	33 (41)	29 (69)	19 (45)
Oral Medications	28 (35)	**38 (48)**	13 (31)	**17 (40)**
Insulin	2 (2)	**9 (11)**	0 (0)	**6 (14)**
Gestational Age at GDM diagnosis (wks)	24.4 (7.5)	22.0 (8.6)	25.0 (7.1)	23.3 (8.1)
Pre-pregnancy BMI, kg/m^2	33.3 (8.3)	33.5 (8.4)	32.6 (7.5)	33.1 (7.6)
Postpartum 6-9 weeks BMI, kg/m^2	33.2 (7.8)	33.5 (7.7)	32.4 (6.6)	33.3 (7.6)
Hypertension history, n	16(20)	19 (24)	8 (19)	8 (19)
Family history of diabetes, n	42 (53)	45 (56)	19 (33)	**27 (64)***
6-9 weeks Postpartum, Lifestyle				
Smoker, n	2 (3)	4 (5)	1 (2)	1 (2)
Physical activity, met-hrs/week	47.4 (21.0)	54.2 (25.1)	49.4 (21.6)	48.8 (24.9)
Total Energy intake, Kcal/day	811 (319)	805 (338)	774 (340)	900.4 (297)
Lactation Intensity Groups, n				
Exclusive lactation	20 (25)	10 (12)	8 (19)	8 (19)
Mostly lactation	30 (38)	28 (35)	15 (36)	17 (41)
Mostly formula/Mixed	18 (22)	19 (24)	10 (24)	12 (29)
Exclusive formula	12 (15)	23 (29)	9 (21)	5 (12)
6-9 weeks Postpartum, Plasma				
Fasting glucose (FPG), mg/dl	95 (8.4)	**103 (10.5)***	93.5 (7.8)	**101.4 (11.3)***
2-hr Post 75 g OGTT (2hPG), mg/dl	109 (25.9)	**132 (29.5)***	116 (28.5)	**132 (30.2)***
Fasting insulin, µU/ml	26 (14.8)	**33 (17.7)***	25.6 (12.1)	29.1 (20)
Fasting triglycerides, mg/dl	128 (90.7)	150 (105.2)	134 (79.6)	151.3 (106)
Fasting HDL-C, mg/dl	49 (13.2)	49 (13.0)	51.5 (13.0)	49.4 (10.9)
HOMA-IR	6.1 (3.7)	**8.6 (5.0)***	5.97 (3.0)	7.47 (5.9)
HOMA-B	299 (183)	305 (156)	313 (153)	284 (193)
Post-baseline, 2-Year Follow Up				
Subsequent Birth, n	5 (6)	5 (6)	9 (21)	**2 (5)***
Follow up in months, median (IQR)	22.4 (1.9)	**16.4 (11.6)***	21.8 (2.8)	18.3 (12.5)

Table 1: Baseline (6-9 weeks post-partum) and follow-up characteristics of SWIFT women with GDM in the training and testing set (n=122 pairs). Date presented are Mean (SD) unless otherwise noted or n (%).Plasma values are from the SWIFT database (25). *p <0.05 between incident T2D and non-T2D groups, and †p<0.05 between training and testing sets.

		Non-T2D	Incident T2D		
No	**Metabolites**	**Mean ± SD**	**Mean ± SD**	**Uncorrected *P*-value**	***Corrected *P*-value**
1	2-Aminoadipic acid	1.06 ± 0.44	1.27 ± 0.54	8.02E-03	1.01E-01
2	Gly	311.1 ± 112.63	279.14 ± 71.7	3.38E-02	2.31E-01
3	Ile	46.94 ± 9.09	51.39 ± 11.8	8.30E-03	1.01E-01
4	Leu	115.05 ± 21.79	126.34 ± 29.01	6.05E-03	9.50E-02
5	Thr	141.13 ± 27.78	154.77 ± 43.81	1.99E-02	1.83E-01
6	Trp	66.76 ± 8.31	70.52 ± 10.99	1.57E-02	1.57E-01
7	Tyr	94.82 ± 17.48	106.33 ± 24.51	7.95E-04	**2.23E-02**
8	Val	230.79 ± 35.52	252.44 ± 45.63	1.01E-03	**2.23E-02**
9	xLeu[+]	200.69 ± 29.18	220.64 ± 43.67	8.63E-04	**2.23E-02**
10	Hexoses	4.7 ± 0.51	5.16 ± 0.63	1.13E-06	**1.24E-04**
11	SM (OH) C16:1	2.87 ± 0.69	2.62 ± 0.8	3.87E-02	2.31E-01
12	SM (OH) C22:2	7.13 ± 1.45	6.59 ± 1.83	3.90E-02	2.31E-01
13	SM C18:0	17.21 ± 3.83	15.82 ± 4.19	2.98E-02	2.31E-01
14	SM C18:1	8.91 ± 2.01	7.94 ± 2.21	4.11E-03	7.54E-02
15	SM C20:2	0.42 ± 0.12	0.34 ± 0.12	1.33E-04	**7.33E-03**
16	SM C24:1	26.86 ± 5.52	24.52 ± 6.44	1.47E-02	1.57E-01
17	PC ae C40:5	4.81 ± 1.21	4.36 ± 1.59	4.32E-02	2.31E-01
18	PC ae C42:5	2.27 ± 0.46	2.08 ± 0.59	2.42E-02	2.05E-01
19	PC ae C44:5	1.18 ± 0.25	1.09 ± 0.32	4.47E-02	2.31E-01
20	AC10	0.25 ± 0.08	0.22 ± 0.06	4.63E-02	2.31E-01
21	AC3	0.28 ± 0.08	0.31 ± 0.1	4.55E-02	2.31E-01
22	Palmitoleic acid (C16:1 n9)	2.76 ± 0.96	2.45 ± 0.86	3.86E-02	2.31E-01

Table 2: Metabolites significantly differ in incident T2D in the training set (n=80 pairs) and concentrations given in µM, except for hexoses, which is provided in mM. **p* values are corrected for multiple comparisons with the Benjamini-Hochberg method and significant metabolites were highlighted in bold text. [+]Metabolise was assayed using both Biocrates plate technology and in-house method but xleu was excluded for prediction analysis. AC, acylcarnitines; Gly, glycine; Ile, isoleucine; Leu, leucine; Thr, threonine; Try, tryptophan; Try, tyrosine; Val, valine; xLeu, xleucine; PC, phosphatidylcholine; SM, sphingolipids.

Sets	Parameters	Optimized Machine learner Algorithm	AUC	Sensitivity	Specificity	Accuracy	Precision	F-score	Best model Score (F score + AUC)
Training	FPG	LR	0.724 (0.645-0.803)	60.00%	75.00%	67.50%	70.60%	64.90%	1.373
	2hPG	LR	0.726 (0.648-0.804)	58.75%	72.50%	65.63%	68.12%	63.09%	1.3569
	Metabolite model	DT	0.830 (0.765-0.894)	86.30%	68.80%	77.50%	73.40%	79.30%	1.623
Testing	FPG	LR	0.706 (0.596-0.816)	57.10%	66.70%	61.90%	63.20%	60.00%	1.306
	2hPG Model	LR	0.661 (0.543-0.779)	57.10%	71.40%	64.30%	66.70%	61.50%	1.276
	Metabolite model	DT	0.769 (0.667-0.871)	73.80%	69.10%	71.40%	70.50%	72.10%	1.490
	Glucose model (FPG and 2hPG)	DT	0.732	88.10%	47.60%	67.90%	62.70%	73.30%	1.465
	Combined model	NB	0.754	54.80%	76.20%	65.50%	69.70%	61.30%	1.367

Table 3: Comparison of FPG, 2hPG and metabolites optimized machine learning performance, indicating greatest performance in the metabolite model. Data presented in mean and (95% CI). LR: Logistic regression, DT: J48 Decision tree NB: Naïve Bayes.

Parameter & metabolite	BMI (kg/m^2)	Fasting Glucose (mg/dl)	2-hr Post 75g OGTT (Glucose mg/dl)	Fasting Insulin (µU/ml)	HOMA-IR
2-AAA	0.210**	0.283***	0.115	0.335***	0.353***
Gly	-0.151+	-0.192*	-0.173*	-0.279***	-0.281***
Ile	0.230**	0.278***	0.144	0.415***	0.437***
Leu	0.055	0.242**	0.15*	0.343***	0.367***
Thr	0.218**	0.156*	0.025	0.150+	0.153+
Trp	-0.161*	0.22**	0.061	0.171*	0.187*
Tyr	0.205**	0.252**	0.028	0.335***	0.353***
Val	0.073	0.235**	0.161*	0.409***	0.418***
AC10	-0.022	-0.165*	0.139	-0.201*	-0.202*
AC3	0.104	0.306***	0.184*	0.362***	0.387***
xLeu+	0.118	0.311***	0.197*	0.481***	0.508***
Hexoses	0.16*	0.826***	0.211**	0.311***	0.429***
Palmitoleic acid (C16:1n9)	0.246**	-0.1	-0.009	0.098	0.068
PC ae C40:5	-0.252**	-0.054	0.081	-0.329***	-0.311***
PC ae C42:5	-0.115	-0.033	0.018	-0.266***	-0.252**
PC ae C44:5	-0.006	-0.177*	-0.182*	-0.204**	-0.217**
SM C18:0	-0.181*	-0.150*	0.028	-0.266***	-0.272***
SM C18:1	-0.049	-0.157*	-0.039	-0.254**	-0.263***
SM C20:2	-0.092	-0.284***	-0.122	-0.358***	-0.376***
SM C24:1	-0.277***	-0.246**	-0.025	-0.475***	-0.475***
SM (OH) C14:1	-0.136	-0.207*	-0.175*	-0.257**	-0.279***
SM (OH) C16:1	-0.161*	-0.199*	-0.087	-0.315***	-0.329***
SM (OH) C22:2	-0.201*	-0.226**	-0.034	-0.378***	-0.385***

Table 4: Pearson correlation coefficients (r) between 22 metabolites that significantly differ in incident T2D compared to non-T2D, as well as metabolite selected by machine learning (SM (OH) C14:1), in the training set (80 pairs) at baseline and clinical parameters BMI, fasting glucose, 2-hour post-load glucose, fasting insulin and HOMA-IR at baseline. $^{+}p=0.05$, $*p<0.05$, $**p<0.005$, $***p<0.0005$.

Figure 1: Study design and metabolic assay work flow

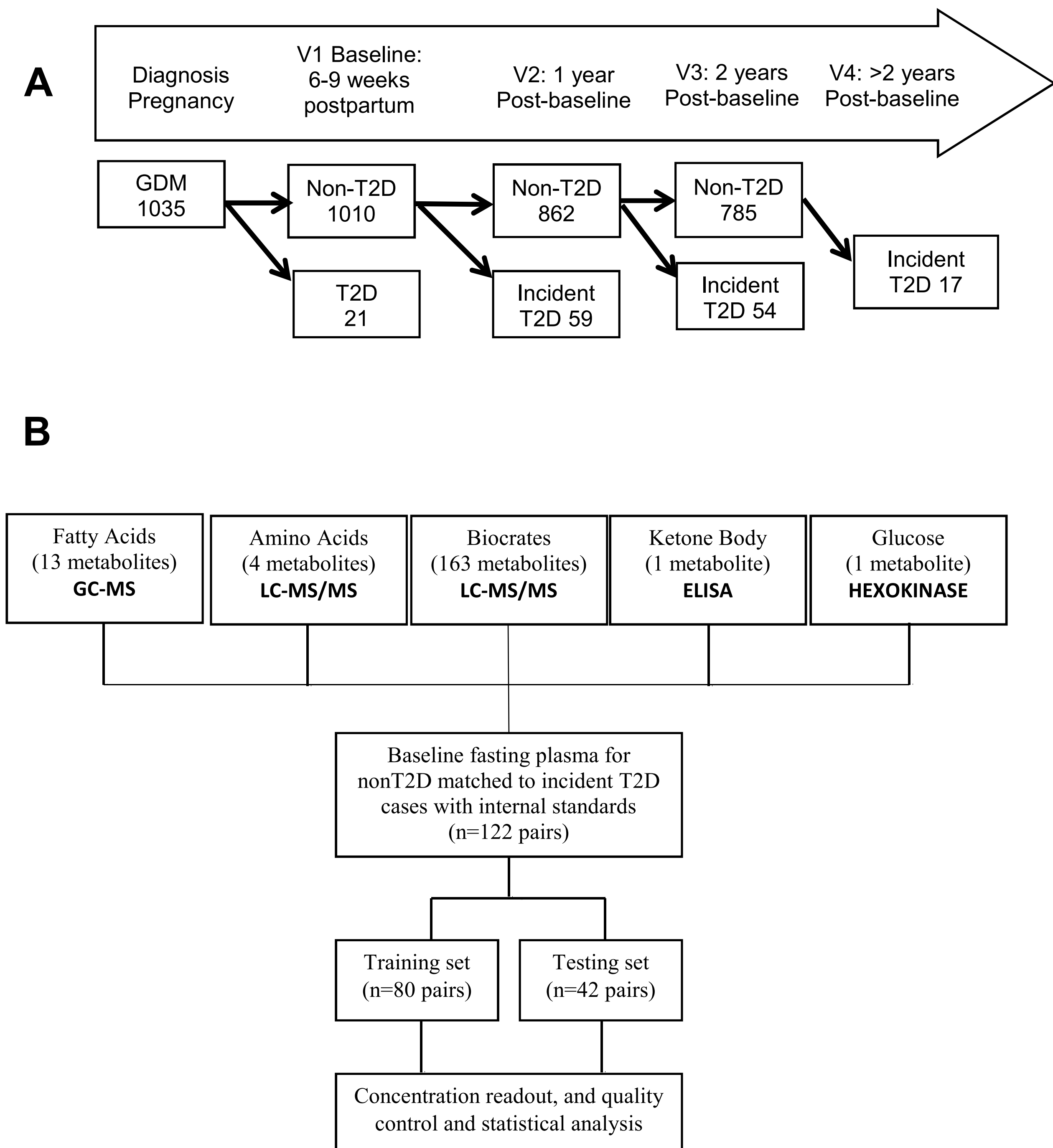
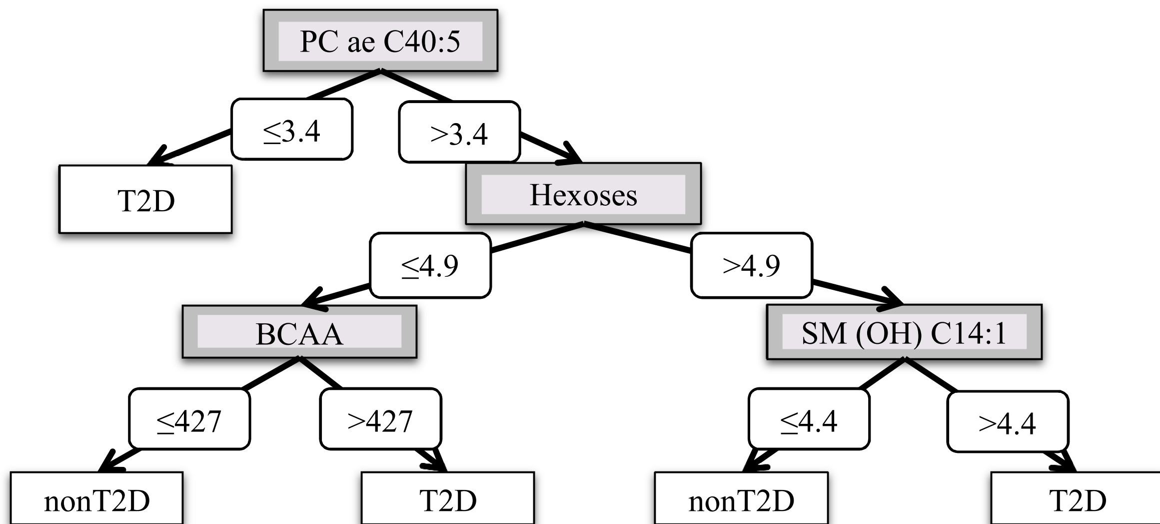


Figure 2: Decision tree and ROC for the prediction of Incident T2D.



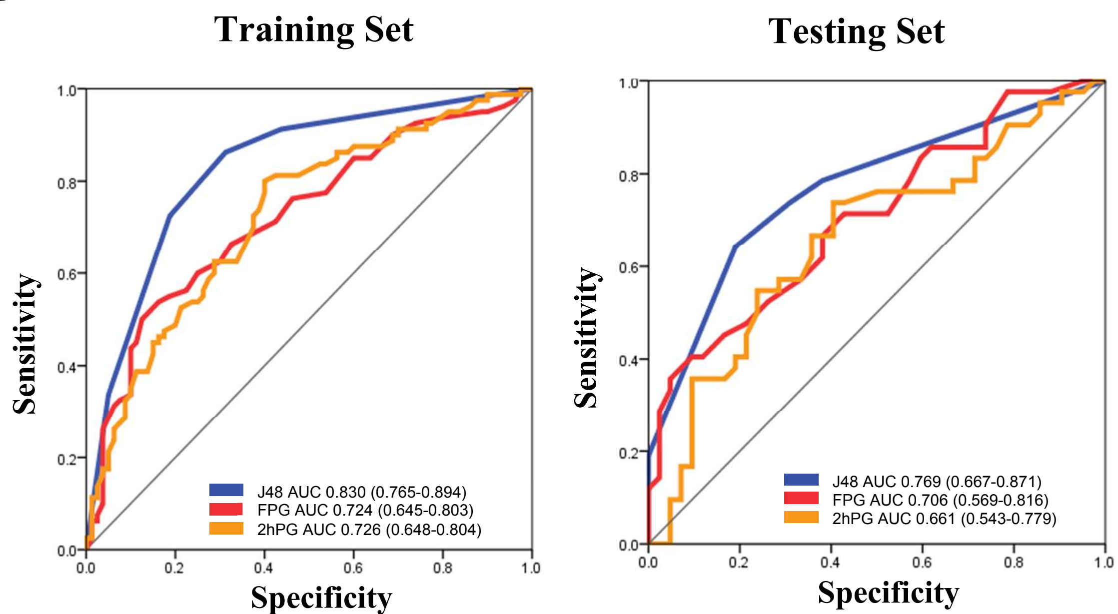
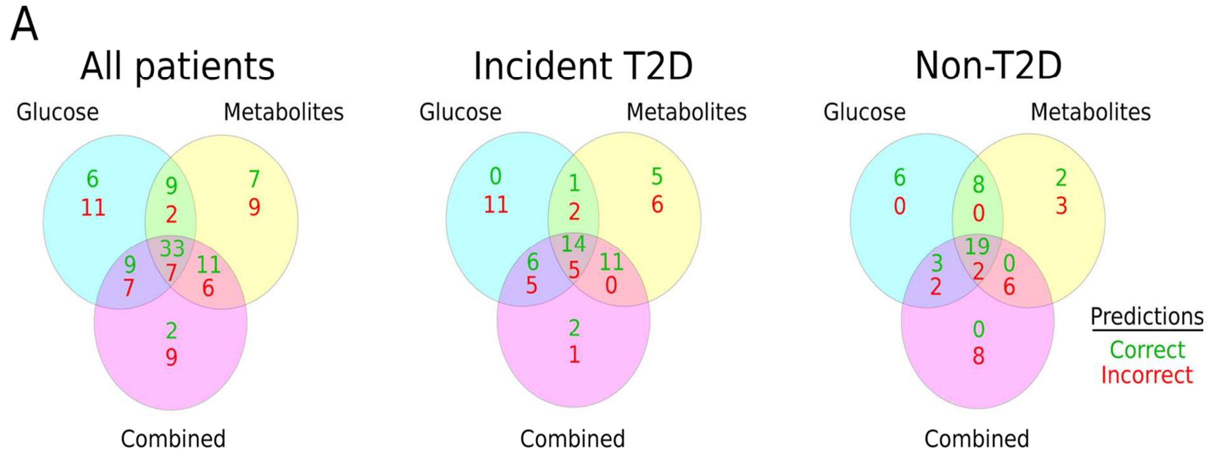
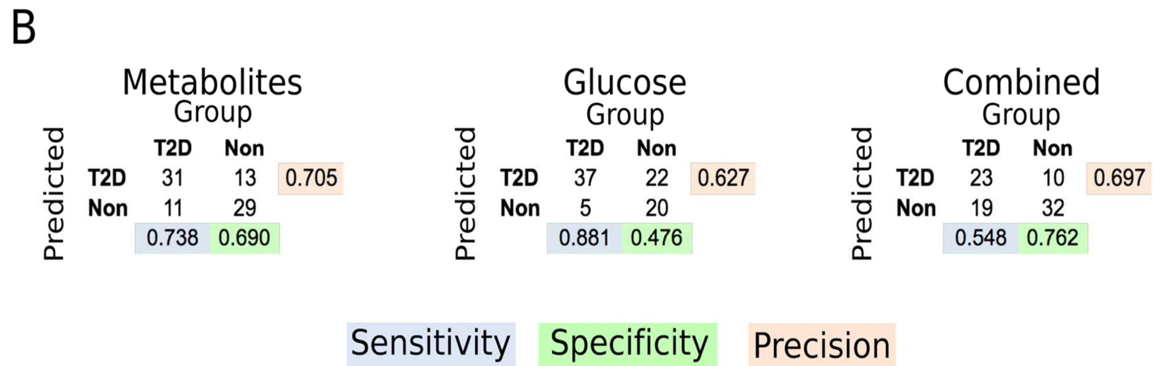


Figure 3: Venn diagrams and contingency tables comparison of model predictions of future diabetes.





B

Metabolites (Predicted vs. Group)

Predicted	T2D	Non	
T2D	31	13	0.705
Non	11	29	
	0.738	0.690	

Glucose (Predicted vs. Group)

Predicted	T2D	Non	
T2D	37	22	0.627
Non	5	20	
	0.881	0.476	

Combined (Predicted vs. Group)

Predicted	T2D	Non	
T2D	23	10	0.697
Non	19	32	
	0.548	0.762	

Sensitivity Specificity Precision

Online Supplemental Materials
Results
Sup. Table 1: Mean and standard deviation of non-T2D and incident T2D cases, uncorrected *p*-value (2-tailed t-test) and corrected *p*-values for multiple comparisons with the Benjamini-Hochberg method of the 110 metabolites that passed all quality control tests (n= 80 pairs, training set) and concentrations given in µM, except for hexoses, which is provided in mM. [+]Metabolise was assayed using both Biocrates plate technology and in-house method but xleu was excluded for prediction analysis. AC, acylcarnitines; Arg, arginine, Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; Met, methionine; Orn, ornithine; PAG, phenyl acetyl glutamine; Phe, phenylalanine; Pro, proline; Ser, serine; SM, sphingolipids; Thr, threonine; Try, tryptophan; Try, tyrosine; Val, valine; xleu, xleucine.

		Non-T2D	**Incident T2D**		
No	**Metabolites**	**Mean ± SD**	**Mean ± SD**	**Uncorrected *P*-value**	**Corrected *P*-value**
1	2-Aminoadipic acid	1.06 ± 0.44	1.27 ± 0.54	**8.02E-03**	1.01E-01
2	Arg	99.72 ± 21.95	105.52 ± 18.95	7.57E-02	2.99E-01
3	Gln	511.29 ± 95.68	514.28 ± 105.81	8.52E-01	9.19E-01
4	Gly	311.1 ± 112.63	279.14 ± 71.7	**3.38E-02**	2.31E-01
5	His	81.54 ± 9.5	83.85 ± 12.61	1.93E-01	4.70E-01
6	Ile	46.94 ± 9.09	51.39 ± 11.8	**8.30E-03**	1.01E-01
7	Leu	115.05 ± 21.79	126.34 ± 29.01	**6.05E-03**	9.50E-02
8	Met	31.16 ± 5.2	32.25 ± 5.25	1.88E-01	4.70E-01
9	Orn	73.75 ± 31.53	75.46 ± 18.29	6.76E-01	8.53E-01
10	Phe	58.91 ± 8.29	60.67 ± 9.14	2.04E-01	4.70E-01
11	PAG	2.2 ± 1.17	2.04 ± 1.19	3.96E-01	6.41E-01
12	Pro	187.58 ± 50.64	190.17 ± 50	7.45E-01	8.53E-01
13	Ser	117.55 ± 24.69	114.93 ± 20.52	4.66E-01	6.83E-01
14	Thr	141.13 ± 27.78	154.77 ± 43.81	**1.99E-02**	1.83E-01
15	Trp	66.76 ± 8.31	70.52 ± 10.99	**1.57E-02**	1.57E-01
16	Tyr	94.82 ± 17.48	106.33 ± 24.51	**7.95E-04**	**2.23E-02**
17	Val	230.79 ± 35.52	252.44 ± 45.63	**1.01E-03**	**2.23E-02**

18	xLeu+	200.69 ± 29.18	220.64 ± 43.67	**8.63E-04**	**2.23E-02**
19	Hexoses	4.7 ± 0.51	5.16 ± 0.63	**1.13E-06**	**1.24E-04**
20	SM (OH) C14:1	5.4 ± 1.24	5.06 ± 1.55	1.29E-01	4.29E-01
21	SM (OH) C16:1	2.87 ± 0.69	2.62 ± 0.8	**3.87E-02**	2.31E-01
22	SM (OH) C22:1	9.9 ± 2.23	9.67 ± 2.62	5.62E-01	7.63E-01
23	SM (OH) C22:2	7.13 ± 1.45	6.59 ± 1.83	**3.90E-02**	2.31E-01
24	SM (OH) C24:1	0.94 ± 0.24	0.9 ± 0.26	3.25E-01	5.68E-01
25	SM C16:0	83.93 ± 12.88	79.38 ± 17.72	6.50E-02	2.83E-01
26	SM C16:1	13.54 ± 1.9	12.89 ± 3.06	1.03E-01	3.55E-01
27	SM C18:0	17.21 ± 3.83	15.82 ± 4.19	**2.98E-02**	2.31E-01
28	SM C18:1	8.91 ± 2.01	7.94 ± 2.21	**4.11E-03**	7.54E-02
29	SM C20:2	0.42 ± 0.12	0.34 ± 0.12	**1.33E-04**	**7.33E-03**
30	SM C24:0	14.51 ± 3.43	14.12 ± 3.6	4.92E-01	7.01E-01
31	SM C24:1	26.86 ± 5.52	24.52 ± 6.44	**1.47E-02**	1.57E-01
32	LPC a C16:0	168.25 ± 32.25	171.54 ± 36.35	5.45E-01	7.50E-01
33	LPC a C16:1	4.44 ± 1.11	4.24 ± 1.03	2.23E-01	4.77E-01
34	LPC a C17:0	3.54 ± 0.98	3.33 ± 0.96	1.77E-01	4.70E-01
35	LPC a C18:0	64.1 ± 14.75	66.94 ± 16.2	2.49E-01	4.89E-01
36	LPC a C18:1	30.48 ± 7.99	29.18 ± 7.05	2.76E-01	4.97E-01
37	LPC a C18:2	38.64 ± 10.79	39.22 ± 11.58	7.45E-01	8.53E-01
38	LPC a C20:3	3.78 ± 1.18	4.01 ± 1.25	2.40E-01	4.89E-01
39	LPC a C20:4	11.81 ± 3.11	11.92 ± 3.98	8.42E-01	9.17E-01
40	PC aa C28:1	3.46 ± 0.78	3.41 ± 1	7.28E-01	8.53E-01
41	PC aa C30:0	3.7 ± 1.16	3.96 ± 1.25	1.78E-01	4.70E-01
42	PC aa C32:0	13.7 ± 2.94	13.69 ± 3.11	9.79E-01	9.88E-01
43	PC aa C32:1	13.04 ± 6.37	14.35 ± 6.69	2.07E-01	4.70E-01
44	PC aa C32:2	5.05 ± 1.9	5.42 ± 1.92	2.30E-01	4.77E-01
45	PC aa C34:1	149.54 ± 31.4	150.14 ± 30.52	9.03E-01	9.55E-01
46	PC aa C34:2	271.39 ± 32.99	277.49 ± 30.96	2.30E-01	4.77E-01

47	PC aa C34:3	17.29 ± 4.55	17.04 ± 4.14	7.15E-01	8.53E-01
48	PC aa C34:4	1.97 ± 0.6	2.1 ± 0.64	1.90E-01	4.70E-01
49	PC aa C36:1	43.92 ± 12.14	45.17 ± 11.09	4.97E-01	7.01E-01
50	PC aa C36:2	200.48 ± 29.03	206.65 ± 31.14	1.97E-01	4.70E-01
51	PC aa C36:3	126.8 ± 25.99	128.65 ± 25.22	6.50E-01	8.41E-01
52	PC aa C36:4	162.87 ± 30.11	164.56 ± 34	7.40E-01	8.53E-01
53	PC aa C36:5	16.1 ± 5.56	17.81 ± 11.1	2.19E-01	4.77E-01
54	PC aa C36:6	0.8 ± 0.27	0.84 ± 0.45	4.22E-01	6.55E-01
55	PC aa C38:0	2.65 ± 0.73	2.65 ± 0.83	9.88E-01	9.88E-01
56	PC aa C38:3	50.18 ± 14.51	53.05 ± 13.96	2.04E-01	4.70E-01
57	PC aa C38:4	101.75 ± 21.63	104.65 ± 24.8	4.32E-01	6.55E-01
58	PC aa C38:5	46.29 ± 10.53	46.41 ± 12.09	9.49E-01	9.84E-01
59	PC aa C38:6	51.86 ± 19.74	50.25 ± 19.32	6.02E-01	7.98E-01
60	PC aa C40:2	0.46 ± 0.16	0.44 ± 0.12	2.06E-01	4.70E-01
61	PC aa C40:4	3.75 ± 1.23	3.99 ± 1.19	2.03E-01	4.70E-01
62	PC aa C40:5	8.91 ± 2.89	9.26 ± 2.47	4.07E-01	6.49E-01
63	PC aa C40:6	18.22 ± 6.87	18.41 ± 6.93	8.64E-01	9.23E-01
64	PC aa C42:0	0.52 ± 0.11	0.5 ± 0.15	2.02E-01	4.70E-01
65	PC aa C42:6	0.46 ± 0.1	0.47 ± 0.1	8.34E-01	9.17E-01
66	PC ae C32:1	2.82 ± 0.59	2.66 ± 0.64	9.93E-02	3.52E-01
67	PC ae C34:0	1.33 ± 0.38	1.32 ± 0.38	8.11E-01	9.17E-01
68	PC ae C34:1	8.85 ± 1.93	8.26 ± 1.86	5.02E-02	2.32E-01
69	PC ae C34:2	13.07 ± 3.35	12.85 ± 4.03	7.10E-01	8.53E-01
70	PC ae C34:3	9.01 ± 3.14	8.45 ± 3.32	2.73E-01	4.97E-01
71	PC ae C36:1	9.96 ± 2.3	9.47 ± 2.49	2.02E-01	4.70E-01
72	PC ae C36:2	15.2 ± 3.52	14.16 ± 3.89	7.80E-02	2.99E-01
73	PC ae C36:3	8.26 ± 2.35	7.97 ± 2.37	4.27E-01	6.55E-01
74	PC ae C36:4	17.94 ± 4.1	17.97 ± 5.84	9.68E-01	9.88E-01
75	PC ae C36:5	10.55 ± 2.76	10.74 ± 4.24	7.30E-01	8.53E-01

76	PC ae C38:4	12.65 ± 2.62	12.04 ± 3.21	1.90E-01	4.70E-01
77	PC ae C38:5	15.34 ± 3.05	15.21 ± 4.29	8.27E-01	9.17E-01
78	PC ae C38:6	6.54 ± 1.64	6.4 ± 2.06	6.40E-01	8.38E-01
79	PC ae C40:2	2.22 ± 0.49	2.06 ± 0.55	5.05E-02	2.32E-01
80	PC ae C40:4	3.34 ± 0.78	3.08 ± 0.96	6.68E-02	2.83E-01
81	PC ae C40:5	4.81 ± 1.21	4.36 ± 1.59	**4.32E-02**	2.31E-01
82	PC ae C40:6	3.45 ± 0.78	3.31 ± 1.11	3.33E-01	5.72E-01
83	PC ae C42:5	2.27 ± 0.46	2.08 ± 0.59	**2.42E-02**	2.05E-01
84	PC ae C44:3	0.313 ± 0.08	0.306 ± 0.08	5.89E-01	7.90E-01
85	PC ae C44:4	0.43 ± 0.09	0.4 ± 0.1	8.19E-02	3.00E-01
86	PC ae C44:5	1.18 ± 0.25	1.09 ± 0.32	**4.47E-02**	2.31E-01
87	PC ae C44:6	1.04 ± 0.25	0.97 ± 0.31	1.53E-01	4.70E-01
88	AC0	37.12 ± 7.85	37.63 ± 10.17	7.22E-01	8.53E-01
89	AC10	0.25 ± 0.08	0.22 ± 0.06	**4.63E-02**	2.31E-01
90	AC2	5.5 ± 1.53	5.22 ± 1.6	2.62E-01	4.91E-01
91	AC3	0.28 ± 0.08	0.31 ± 0.1	**4.55E-02**	2.31E-01
92	AC4	0.18 ± 0.07	0.18 ± 0.06	4.75E-01	6.88E-01
93	AC5	0.098 ± 0.028	0.102± 0.030	3.09E-01	5.47E-01
94	AC8:1	0.17 ± 0.07	0.16 ± 0.07	8.20E-01	9.17E-01
95	AC18:1	0.090 ± 0.024	0.086 ± 0.025	3.73E-01	6.27E-01
96	AC18:2	0.04 ± 0.012	0.04 ± 0.012	9.82E-01	9.88E-01
97	Myristic acid (C14:0)	11.49 ± 4.21	10.66 ± 4.79	2.48E-01	4.89E-01
98	Palmitic acid (C16:0)	203.14 ± 78	197.8 ± 89.91	6.91E-01	8.53E-01
99	Hexadecenoic acid (C16:1 n-7)	21.94 ± 8.78	20.48 ± 11.7	3.76E-01	6.27E-01
100	Palmitoleic acid (C16:1 n-9)	2.76 ± 0.96	2.45 ± 0.86	**3.86E-02**	2.31E-01
101	Stearic aicd (C18:0)	43.22 ± 25.11	46.52 ± 36.3	5.09E-01	7.09E-01
102	Oleic Acid & Vaccenic Acid (C18:1 n-9, n-7)	284.9 ± 143.69	264.78 ± 143.65	3.82E-01	6.27E-01
103	Linoleic acid (C18:2)	197.52 ± 77.79	183.65 ± 74.2	2.55E-01	4.91E-01
104	Alpha-linolenic acid (C18:3)	8.56 ± 3.95	8.52 ± 4.23	9.46E-01	9.84E-01

105	Eicosenoic acid (C20:1)	1.46 ± 0.61	1.36 ± 0.55	2.63E-01	4.91E-01
106	Arachidonic acid (C20:4)	16.39 ± 7.82	17.02 ± 14.27	7.33E-01	8.53E-01
107	Eicosapentaenoic acid (C20:5)	1.72 ± 0.99	2.17 ± 3	2.09E-01	4.70E-01
108	Docosapentaenoic acid (C22:5)	0.93 ± 0.4	1 ± 0.71	4.35E-01	6.55E-01
109	Docosahexaenoic acid C22:6)	3.86 ± 2.33	4.29 ± 4.71	4.63E-01	6.83E-01
110	Beta-hydroxybutyrate	137.35 ± 93.18	172.76 ± 151.56	7.89E-02	2.99E-01