GC-MS based Gestational Diabetes Mellitus longitudinal study

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Abstract

Gestational Diabetes Mellitus (GDM) causes severe short- and long-term complications for the mother, fetus and neonate, including type 2-diabetes (T2DM) later in life.

In this pilot study, GC-Q/MS analysis was applied for plasma metabolomics fingerprinting of 24 healthy and 24 women with GDM at different stages of gestation (second and third trimester) and postpartum (one and three months). Multivariate (unsupervised and supervised) statistical analysis was performed to investigate variance in the data, identify outliers and for unbiased assessment of data quality.

Plasma fingerprints allowed for the discrimination of GDM pregnant women from controls both in the 2nd and 3rd trimesters of gestation. However, metabolic profiles tended to be similar after delivery. Follow up of these women revealed that 4 of them developed T2DM within 2 years postpartum. Multivariate PLS-DA models limited to women with GDM showed clear separation 3 months postpartum. In the 2nd trimester of gestation there was also a clear separation between GDM women that were normoglycemic after pregnancy and those with recognized postpartum T2DM.

Metabolites that had the strongest discriminative power between these groups in the 2nd trimester of gestation were 2-hydroxybutyrate, 3-hydroxybutyrate, and stearic acid. We have described, for the first time, that early GDM comprises metabotypes that are associated with the risk of future complications, including postpartum T2DM. In this pilot study, we provide evidence that 2-hydroxybutyrate and 3-hydroxybutyrate may be considered as future prognostic biomarkers to predict the onset of diabetic complications in women with gestational diabetes after delivery.

Keywords: Gestational diabetes mellitus; maternal metabolism; fingerprinting; type 2-diabetes mellitus; gas chromatography; metabolomics.

Abbreviations: ANOVA: analisis of variance; BMI: body mass index; CRP: C-reactive protein; EI: electron ionization; FDR: false discovery rate; GC: gas chromatography; GC-MS: gas chromatography couple with mass spectrometry; GC-EI-MS-Q: gas chromatography-electrospray ionization single quadrupole mass spectrometry; GDM: gestational diabetes mellitus; HbA1c: glycated haemoglobin; HDL: high density lipoprotein; HOMA-IR: homoeostasis model assessment-insulin resistance; LC-MS: liquid chromatography couple with mass spectrometry LDL: low density lipoprotein; MS: mass spectrometry; OGTT: oral glucose tolerance test; PLS-DA: partial least squares discriminant analysis; QA: quality assurance; QC: Quality control; QUICKI: quantitative insulin sensitivity check index; RSD: relative standard deviation; RI: retention indexes; ROC: receiver operator curve; RT: retention time; RTL: Retention Time Locked; T2DM: type 2-diabetes mellitus.

1 Introduction

Gestational Diabetes Mellitus (GDM), defined as "any degree of glucose intolerance with onset or first recognition during pregnancy"[1], is increasing worldwide and it is expected to further rise as the epidemic of obesity continues. Despite advances in diagnosis and good maternal control [2], GDM is associated with short- and long-term complications for both the mother and the offspring, including caesarean and operative vaginal delivery, maternal pre-eclampsia, or newborn macrosomia, shoulder dystocia, hypoglycemia or hyperbilirubinemia [3, 4]. Furthermore, although GDM usually remits shortly after delivery, these women have a high risk of developing postpartum glycemic alterations, such as glucose intolerance or even type 2-diabetes mellitus (T2DM) [5, 6, 7]. Although this association is well established, the magnitude of the risk varies among different studies. Generally, this has been explained by differences in the diagnostic criteria and the design of the study (e.g. selection and number of the participants, length of follow-up) [8]. Thus, women with GDM should be followed up after parturition to allow detection of early development of T2DM. In this scenario, it is of interest to detect women at a higher risk of future T2DM before delivery and, if possible, at the time of GDM diagnosis. Therefore, a better understanding of the pathophysiology of GDM as well as the identification of potentially early diagnostic markers for GDM, are one of the most relevant health issues.

Current "omics" techniques, in particular metabolomics, provide deeper insights into disease-related metabolic alterations and etiopathogenesis of the diseases and, accordingly, are useful in biomarker discovery. In fact, the approaches to translate basic metabolomics into clinical applications are increasing. A growing number of metabolomics studies, aimed at uncovering the metabolic signature of T2DM [9,10], focus on potential biomarkers of altered glucose tolerance and onset of insulin resistance.

151 Despite disparities in predictive biomarkers [11], metabolomics studies have the potential
152 to determine sets of metabolites that are predictive of both prediabetes and T2DM, even
153 before the onset of disease, thereby improving patients' health, as shown recently for
154 T2DM [12]. In fact, an increasing number of studies have confirmed elevation of
155 circulating branched-chain amino acids and 2-hydroxybutyrate before manifestation of
156 T2DM. On the contrary, glycine and lysophosphatidylcholine C18:2 concentrations were
157 found to be decreased in both predictive studies and with overt disease [11, 13]. Thus,
158 although there is much work left to do, the evidence of metabolomics benefitting T2DM
159 care makes its clinical application inevitable, and this can be extended to GDM.
160 The first multi-platform, non-targeted metabolome wide analyse in plasma and urine of
161 GDM was presented in our previous study [14]. We found that, in the 2nd trimester of
162 gestation, plasma metabolite fingerprints revealed metabolic imbalances and proposed a
163 comprehensive picture of the early metabolic alterations in GDM. In particular, we
164 provided evidence for the implication of some compounds, as 2-hydroxybutyrate,
165 glycine, lysophosphatidylcholine (18:2), and other lysophospholipids, in metabolic routes
166 that may be associated with the early genesis of GDM, which highlights their potential
167 use as prognostic markers for the identification of women at risk to develop severe
168 glucose intolerance during pregnancy [14]. However, up to now it has not been analyzed
169 whether there are differences in the metabolic profiles of those women at higher risk of
170 T2DM after delivery.
171 Based on these findings, we propose that in GDM there are different metabotypes
172 associated with further post-partum glycemic alterations that can be detected by
173 metabolomics. To corroborate this hypothesis, we devised a metabolomics approach to
174 obtain a picture of metabolic profiles during and after pregnancy, with the ultimate goal

to identify metabotypes of GDM and to eventually identify potential biomarkers that predict the risk of GDM pregnant women to develop T2DM after delivery.

2 Materials and methods

2.1 Experimental design and Study Population

All participants were recruited in the Public Clinic Hospital, Medical University of Bialystok (Poland). Women having overt diabetes mellitus or other complications were excluded from the study. At the initiation of the study the population consisted of total 68 participants, matched according to week of gestation and age (22-37 years). Screening of GDM was performed at 22-28 weeks of gestation after overnight fasting by an oral glucose tolerance test (OGTT). GDM was defined, according to WHO-1998 criteria, as glucose level ≥140 mg/dl (7.8 mmol/L) after 2-h 75-g OGTT. After GDM diagnosis, the control and GDM groups included 37 healthy pregnant women and 31women with GDM, respectively. Fasting blood samples were collected in into EDTA containing tubes at different times: 2^{nd} (at the day of the OGTT) and 3^{rd} trimester of gestation, and 1 month and 3 months after delivery. When 2 or more time-samples were missing, the woman was excluded from the analysis. Finally, 24 control and 24 cases were included in the study. Women diagnosed with GDM were followed for two years after delivery.

The study was carried out in accordance with the permission of the Bioethical Commission of the Medical University of Bialystok. Written informed consent was obtained from each participant in the study.

2.2 Biochemical analysis and indexes of insulin resistance

Plasma glucose, cholesterol, LDL/HDL-cholesterol, triacylglycerols and C-reactive protein (CRP) were measured in an autoanalyzer (Cobas C111 Roche Autoanalyzer, Hoffmann-LaRoche Ltd., Basel Switzerland). Blood HbA1c was analyzed by the D-10TM Hemoglobin Testing System (Bio-Rad, USA), C-peptide by an ELISA kit

(Biosource International, Inc., Belgium), and insulin with an INS-IRMA-RIA kit (DIAsource ImmunoAssays S.A., Belgium). HOMA-IR (homoeostasis model assessment-insulin resistance) [15] and QUICKI (quantitative insulin sensitivity check index) [16] indexes were calculated with fasting glucose (mg/dL) and insulin (μU/mL) as described.

2.3 Chemicals and reagents

Standard mix for GC-MS, containing grain fatty acid methyl ester mixture (C8:0-C22:1n9), and LC-MS grade organic solvents, acetonitrile, 2-propanol and analytical grade heptane were from Fluka Analytical (Sigma-AldrichChemie GmbH, Steinheim, Germany). C18:0 methyl ester, N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane were from (Pierce Chemical Co, Rockford, IL, USA). Silylation grade pyridine was from VWR International BHD Prolabo (Madrid, Spain).

2.4 Sample preparation

Metabolic extracts from plasma were prepared for analysis as previously described [14]. Plasma (50 μL) protein was precipitated with cold acetonitrile (150 μL) and separated by centrifugation (15400g, 10min, 4°C). The resulting supernatant was transferred to GC vial with insert and then evaporated to dryness (Speedvac Concentrator, Thermo Fisher Scientific, Waltham, MA, USA). Ten microliters (10 μL) of O-methoxyamine hydrochloride in pyridine (15 mg/mL) was added to each GC vial, and mixture was vigorously vortex-mixed and ultrasonicate. Methoxymation was carried out in darkness, at room temperature for 16 h. BSTFA with 1% TMCS (10 μL) was then added as catalyst. For silylation process samples were heated in an oven for 1 h at 70 °C. Finally, 100 μL of heptane containing 10 ppm of C18:0 methyl ester (IS) was added to each GC vial and vortex-mixed before GC analysis. Quality control (QC) samples were prepared by

pooling equal volumes of each sample and were subjected to identical extraction procedures as the experimental samples.

GC-EI-Q-MS analysis

GC-MS analyses were performed by a GC system (Agilent Technologies 7890A) equipped with an autosampler (Agilent 7693) coupled to a mass spectrometer with triple-Axis detector (5975C, Agilent). Two microliters (2 μL) of the derivatized sample were injected through a GC-Column DB5-MS (30 m length, 0.25 mm i.d., 0.25 μm film 95% dimethyl/5% diphenylpolysiloxane) with an integrated precolumn (10 m J&W, Agilent). Carrier gas (He) flow rate was set at 1 mL/min and injector temperature at 250 °C. Split ratio was fixed from 1:5 to 1:10 with 3 to 10 mL/min He split flow into a Restek 20782 (Bellefonte, PA, USA) deactivated glass-wool split liner. The temperature gradient was programmed as follows: the initial oven temperature was set at 60 ºC (held for 1 min), increased to 325 ºC at 10 ºC/min rate (within 26.5 min) and hold 325 ºC for 10 min. The total run time was 37.5 min. A cool-down period was applied for 10 min before the next injection. Detector transfer line, filament source and the quadrupole temperature were set at 280 ºC, 230 ºC and 150 ºC, respectively. MS detection was performed with electron ionization (EI) mode at -70 eV. The mass spectrometer was operated in scan mode over a mass range of *m/z* 50-600 at a rate of 2.7 scan/s. Internal standard C18:0 methyl ester (10 ppm), a standard mix of fatty acid methyl esters (FAME C8-C30), extraction blank and 2 QCs samples were injected at the beginning of analysis, following QCs injections every 8 experimental samples and 2 QCs injections at the end of worklist. These conditions were optimized as described previously [17,18].

2.5 Data treatment and compound identification

GC-MS data, peak detection and spectra processing algorithms were applied using the Agilent MSD ChemStation Software (G1701EA E.02.00.493, Agilent). The overall

quality of analytical performance was carefully examined by inspection of total ion chromatograms (TIC) of experimental samples, QC samples, blanks and internal standard. Automated Mass Spectrometry Deconvolution and Identification System (AMDIS) version 2.69 software from NIST (U.S. National Institute of Standards and Technology) was used for automatic mass spectral deconvolution to identify co-eluted compounds according to their retention indexes (RI) and retention times (RT). Retention times from analysis of fatty acid methyl ester standard solution was used to create a calibration data file for further adjustment of RT in samples. The accuracy improvement was based on the determination of the Kovats RI. Retention index value, contained in Fiehn RTL (Retention Time Locked) library was compared to the experimental RI value in order to assign a match score between the experimental and the theoretical spectra. Compounds were identified by comparing their mass fragmentation patterns with target metabolite Fiehn GC/MS Metabolomics RTL library (G1676AA, Agilent), the in-house CEMBIO-library and the NIST mass spectra library 2.0, using the ChemStation software and native PBM (Probability-Based Matching) algorithm (G1701EA E.02.00.493, Agilent). Alignment of drift (by retention time and mass) and data filtering were performed with the Mass Profiler Professional B.12.1 (Agilent) software. Variation of the compounds abundance in QC samples, expressed as relative standard deviation (%RSD), was also calculated. To limit results to metabolites with good repeatability, those features detected in <50% of all QC samples and with a RSD >30% in QC samples were removed. Data matrix was normalized according to internal standard C18:0 methyl ester intensity.

2.6 Statistical analysis

The sample size was calculated by power analysis (G*Power 3.1.9). According to our previous study [14] we considered 2-hydroxybutyrate as primary variable. Effect size was set at 1.0 and alpha 0.05. According to these values a study with 38 participants has 90%

power. While we finally included 24 controls and 24 GDM pregnant women (48 participants), power increased to 95%. For multivariate (unsupervised and supervised) statistical analysis, the processed data matrix was imported to SIMCA-P+ 14.1 (Umetrics, Umea, Sweden). PCA (principal component analysis), where highly correlated metabolic variables are projected onto a smaller set of linearly uncorrelated variables called principal components [19], was performed to investigate multivariate variance in the data, identified sample outliers and to unbiased assessment of data quality (QCs sample plotting). Validation of partial least squares discriminant analysis (PLS-DA) models were performed by 7-fold cross validation algorithm as described [20]. Variable Importance in Projection (VIP) with VIP>1.0 cutoff and Jack-Knife with confidence intervals estimative, 95% confidence level was applied for the selection of key variables. Prior to univariate statistical analysis, data normality was verified by evaluation of the Kolmogorow−Smirnov−Lillefors and Shapiro−Wilk tests and variance ratio by the Levene's test. Data are shown as mean ± SD or as median and interquartile range according the variable distribution. Differences between two groups were evaluated by paired or unpaired *t* test (equal or unequal variance) or nonparametric (Mann−Whitney test) with post hoc Benajmini-Hochberg (FDR, false discovery rate), and Bonferroni test respectively, for multiple comparisons. One-way ANOVA with repeated measures pairwise comparisons was applied to analyze data during pregnancy and postpartum.

The levels of statistical significance for any statistical test performed, before and after multiple comparison correction were set at 95% level ($P < 0.05$). Statistical analyses were performed using Matlab R2015a. ROC analysis was performed using GraphPad programm (v. 6.0 for Macintosh).

3. Results and discussion

3.1. Study participants

As shown in Table 1, there was no difference in age, parity, and prepregnancy BMI in women participating in the study. In the 2nd trimester of pregnancy, there were no significant differences in blood pressure, BMI, HDL and LDL-cholesterol, insulin, C peptide, CRP, HOMA-IR and QUICKI between the control and the GDM women. However, women classified as GDM had significantly higher fasting glucose, HbA1c and triacylglycerides than controls and, during the OGTT, glucose levels were significantly higher at one and two hours in the women classified as GDM according to WHO-criteria (Table 1). Analysis of clinical data three months after delivery showed no significant differences in BMI, basal glucose, insulin, triacylglicerides, HDL and LDL-cholesterol, CRP, HbA1c, HOMA-IR and QUICKI, although a C-peptide and one hour glucose-OGGT were significantly higher in the women classified as GDM (Table 2). Although these results point to a recovery of GDM after postpartum, the observed differences in one hour glucose-OGTT and C-peptide, suggest that some of the women could have some degree of glucose intolerance after delivery. In fact, at 3 months postpartum, 7 GDM women had altered glycemia. Follow up of the GDM women confirmed that 4 of them were diagnosed as T2DM within two years after delivery.

3.2. Metabolomics analysis

GC–MS based analysis was applied for plasma metabolomics analysis of control and GDM women at different times of gestation, as well as one and three months postpartum. Samples were analysed in randomized order, and quality control samples were included to control system's stability, performance and reproducibility of the sample treatment procedure. After matrix filtration, according to quality assurance (QA) criteria [21], a total of 37 metabolites were considered for further data treatment. For multivariate analysis all variables were log transformed and autoscaled. PCA analysis was performed to investigate multivariate variance in the data, identify sample outliers and for unbiased

assessment of data quality. According to Hotelling's T2 Range based on PCA model, one strong analytical outlier (control group) was detected and excluded from further multivariate and univariate calculations. Additionally, supervised regression method, based on PLS-DA, was used for modelling the differences between disease and control groups. PLS-DA models shown in Fig. 1 and 2 were described with R2 (explained variance) and Q2 (predictive variance) values that represent the quality of the model. First, to explore the evolution of the metabolic profile during pregnancy and after delivery, PLS-DA models were established separately for control and GDM groups at each time point (2^{nd}, 3^{rd} trimester and 1 month and 3 months postpartum). Cross-validation tool was used to validate PLS-DA model based on the data derived from the 2^{nd} trimester of pregnancy, with the value of 78±9% of samples classified correctly. As shown in Fig.1, there was a tendency for group separation between control and GDM women both in the 2^{nd} and 3^{rd} trimester of gestation and after delivery. We observed that, despite the metabolic control of the GDM women during gestation, the metabolic profile of GDM is not fully corrected during late pregnancy. Probably, this could be related to the role of identified compounds (Table 3) in the molecular mechanisms of the disease.

As this longitudinal follow up study provided information of glycemic condition after delivery, we explored whether the metabolic profile was different in women that developed some degree of glucose intolerance or T2DM after delivery, as compared to those with normal glucose tolerance. Multivariate PLS-DA models limited exclusively to GDM women showed clear separation between those that were normoglycemic and those with recognized glycemic alterations at 3 months postpartum (data not shown). Follow up of these women revealed that 4 of them developed T2DM within 2 years postpartum. Multivariate PLS-DA models for those selected cases showed clear separation 3 months postpartum between GDM women that were normoglycemic after pregnancy and those

with postpartum T2DM (Fig.2A), with the value of 88±10% samples classified correctly according to cross validation analysis. To find if these women had already an altered metabolic profile at the time of GDM diagnosis, we performed a retrospective analysis of the metabolic profile in the 2nd trimester of gestation of these post-partum T2DM women. As shown in Fig.2B we observed a strong separation of these at-risk samples, with the 80±15% rate of samples classified positively. Multivariate analysis of metabolites in the 2nd trimester of gestation revealed that 2-hydroxybutyrate and 3-hydroxybutyrate exhibited the stronger variation in those GDM women that 2 years after delivery were T2DM as compared with those that were GDM and normoglycemic after parturition (Table 4). Other compounds, such as stearic acid and other fatty acids, also were significantly different between these groups. Interestingly, we found that these compounds were also good predictors of glycemic alterations 3 months after delivery in GDM women, suggesting that they may constitute relevant etiophatogenic factors that favour or are related with post-partum T2DM in women with previous GDM.

Finally, we performed a ROC analysis with those metabolites that showed the strongest differences between those GDM women with T2DM after pregnancy and those who did not have any alteration of glycemic state after delivery. As shown in Fig.3, 2-hydroxybutyrate, 3-hydroxybutyrate, and stearic acid have the best discriminative power, whereas threitol, oleic, linoleic, palmitic, palmitoleic, and lactic acid did not have an AUC significantly different from 0.5 (data not shown). These results suggest that women with GDM at a higher risk of glycemic alteration after delivery exhibit a distinct metabotype even before the diagnosis of the disease.

Furthermore, although the study should be extended and validated in a higher cohort, our results suggest that analysis of 2-hydroxybutyrate and 3-hydroxybutyrate in the 2nd trimester when the OGTT is performed will help to design a more strict control in those

374 women at higher risk of T2DM after delivery. The obtained results in our study support
375 the role of 2-hydroxybutyrate (an organic acid derived from 2-ketobutyrate) as a relevant
376 predictive biomarker of glycemic alterations, both in human and animal models of type
377 2-diabetes [22, 23, 24, 25]. In particular, different metabolomics studies have described
378 an increase in 2-hydroxybutyric acid up to 9.5 years ahead of T2DM presentation [9, 24],
379 pointing to a role of this compound as an independent and early predictor of glucose
380 intolerance in humans [9, 26]. It is hypothesized that increased lipid oxidation, oxidative
381 stress and enhanced glutathione synthesis [27] might explain the observed differences in
382 2-hydroxybutyrate. Interestingly, in a previous study from our group [14] we found that,
383 in the 2nd trimester of gestation, plasma 2-hydroxybutyrate concentration was higher in
384 GDM than in controls. Since it is known that elevation of 2-hydroxybutyrate may occur
385 *in vivo* when the formation of 2-ketobutyrate exceeds the rate of its catabolism, we
386 proposed that a redox imbalance and glutathione synthesis, consistent with increased fatty
387 acid oxidation, may contribute to elevated 2-hydroxybutyrate in GDM (a graphical model
388 of metabolic alterations in second trimester in GDM is shown in [14]). This switch to
389 fatty acid oxidation is further supported by increased levels of 3-hydroxybutyrate, a
390 ketone body derived from fatty acid oxidation, in GDM women in the 2nd trimester of
391 gestation [14, 28]. Interestingly, elevated levels of 3-hydroxybutyrate have been
392 described 3 years ahead of T2DM manifestation [24]. Furthermore, some studies have
393 shown that circulating stearic acid is associated with higher diabetes risk [29].
394 Different studies have shown that GDM and T2DM share many of the risk factors, such
395 as an increased BMI, age or family history of diabetes [8, 30]. Some of the known T2DM
396 risk genes are also more frequent in women with previous GDM [30, 31]. Thus, it appears
397 plausible that the pathogenesis of GDM and T2DM is overlapping [32]. In this scenario,
398 the observed elevation of 2-hydroxybutyrate, and 3-hydroxybutyrate in GDM, together

399 with significantly higher levels in GDM women that developed 2TDM after parturition,
400 led us to suggest a causal role of these compounds, together with the underlying increased
401 fatty acid oxidation, in the development of the disease and its complications. Thus, we
402 hypothesize that among women that develop GDM, there is a specific metabotype that is
403 more prone to develop T2DM after delivery; 2-hydroxybutyrate and 3-hydroxybutyrate
404 may serve to identify these women, and may be considered both as potential predictive
405 and prognosis biomarkers. Establishing different metabotypes at the time of diagnosis of
406 GDM could provide an opportunity to test and perform dietary, lifestyle, and/or
407 pharmacological interventions that might prevent or delay the onset of T2DM in the
408 women at higher risk.
409 Metabolomics has the potential to determine set of metabolites that are predictive of both
410 prediabetes and T2DM, even before onset of the disease [12]. Thus, the evidence of
411 metabolomics benefitting T2DM, and also GDM patients, makes its clinical application
412 inevitable. Although sample size can be a potential limitation, it should be considered that
413 nontargeted metabolomics studies typically measure hundreds of metabolites, an
414 approach that is not realistic or cost-effective for large-scale application. Thus, pilot
415 studies in metabolomics are of importance before validation on the large cohorts and the
416 final translation into clinical diagnosis. Considering that this is a pilot study, the sample
417 size and the statistical power applied, together with a homogeneous sample set and very
418 strict analytical control, allow us to detect the most relevant associations. Additionally,
419 our study had several strengths. It represents the first metabolomics longitudinal analysis
420 of human GDM, not only during pregnancy but also after parturition. It has the advantage
421 that it is not a cross-sectional study, and every woman was followed up during pregnancy
422 and postpartum, avoiding the effects of cohort. In fact, we used a well-established cohort
423 and a serial assessment of metabolomics analysis, which minimized selection and

ascertainment biases. Together, this supports the value of the obtained results, which can be considered as potential biomarkers that should be further validated in a targeted study.

4. Conclusions

To our knowledge, this study represents the first non-targeted longitudinal metabolome wide analyses in plasma of GDM during pregnancy and postpartum. We show that metabolic control of the patients is associated with a global metabolic improvement, although some metabolites remained altered in GDM patients as compared to controls. Furthermore, we have described for the first time that early GDM comprises metabotypes that are associated with risk of future complications, including postpartum T2DM. As this is a pilot study, future projects including targeted validation in other cohorts and with a higher number of patients will allow us to validate the identified biomarkers (mainly 2-hydroxybutyrate and 3-hydroxybutyrate) as prognostic tools to predict the early onset of diabetic complications in GDM women after delivery.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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577 **Table 1. Anthropometric and metabolic characteristic of the women included in the**

578 **study in the second trimester of gestation.**

Parameter	Control group n = 24	GDM group n = 24	P value
Age (years)	29.2 ± 2.3	28.3 ± 4.2	ns
Parity (number)	1.46 ± 0.66	1.46 ± 0.78	ns
Week of gestation	25.8 ± 1.9	25.6 ± 1.9	ns
Pre-pregnancy BMI (kg/m^2)	22.4 ± 2.5	24.8 ± 5.6	ns
2^{nd} trimester BMI (kg/m^2)	25.4 ± 2.88	27.7 ± 6.0	ns
Fasting glucose (mmol/L)	4.52 ± 0.29	5.09 ± 0.77	0.0003
1-h, OGTT (mmol/L)	7.31 ± 1.53	8.95 ± 1.98	0.0013
2-h, OGTT (mmol/L)	6.37 ± 1.19	9.18 ± 1.0	<0.0001
$HbA1_C$ (%)	4.91 ± 0.33	5.2 ± 0.4	0.0199
Insulin (pmol/L)	93.1 ± 33.7	88.5 ± 40.2	ns
C-Peptide (pmol/mL)	0.57 ± 0.25	0.74 ± 0.8	ns
HOMA-IR	2.52 ± 1.17	2.73 ± 1.49	ns
QUICKI	0.55 ± 0.17	0.55 ± 0.13	ns
Triacylglycerides (mmol/L)	1.68 ± 0.51	2.08 ± 0.65	0.0081
Total cholesterol (mmol/L)	6.44 ± 1.12	6.63 ± 0.74	ns
LDL-cholesterol (mmol/L)	3.37 ± 1.01	3.66 ± 0.93	ns
HDL-cholesterol (mmol/L)	2.37 ± 0.40	2.21 ± 0.45	ns
CRP (μg/mL)	3.95 ± 3.49	5.09 ± 4.25	ns
Systolic BP (mm Hg)	117.0 ± 7.2	115.0 ± 11.3	ns
Diastolic BP (mmg Hg)	72.1 ± 8.4	72.8 ± 9.6	ns

579 Presented data are mean ± SD. Statistical comparisons assuming equal (*t* test) or unequal
580 variance (Welch's *t* test) or non-parametric Mann-Whitney test were performed as
581 appropriate. Results were considered significant when $P < 0.05$.

582 **Table 2. Anthropometric and metabolic characteristic of the women 3 months after**

583 **delivery.**

Parameter	Control group n = 24	GDM group n = 24	P value
3 months postpartum BMI (kg/m^2)	38.1 ± 4.67	41.14 ± 9.76	ns
Fasting glucose (mmol/L)	4.75 ± 0.41	5.53 ± 2.59	ns
1-h, OGTT (mmol/L)	6.24 ± 1.41	9.05 ± 4.42	<0.0001
2-h, OGTT (mmol/L)	5.17 ± 0.94	7.14 ± 6.04	ns
HbA1C (%)	5.23 ± 0.42	5.51 ± 0.83	ns
Insulin (pmol/L)	67.17 ± 30.0	73.22 ± 49.03	ns
C-Peptide (pmol/mL)	0.57 ± 0.30	1.0 ± 0.78	0.0118
HOMA-IR	46.16 ± 21.13	56.6 ± 42.82	ns
QUICKI	0.63 ± 0.08	0.62 ± 0.11	ns
Triacylglycerides (mmol/L)	1.69 ± 1.2	2.37 ± 2.07	ns
Total cholesterol (mmol/L)	4.84 ± 0.83	5.1 ± 0.95	ns
LDL-cholesterol (mmol/L)	2.81 ± 0.75	3.0 ± 1.02	ns
HDL-cholesterol (mmol/L)	1.85 ± 0.5	1.78 ± 0.38	ns
CRP (μg/mL)	2.9 ± 4.45	1.76 ± 1.78	ns

584 Presented data are mean ± SD. Statistical comparisons assuming equal (*t* test) or unequal
585 variance (Welch's *t* test) or non-parametric Mann-Whitney test were performed as
586 appropriate. Results were considered significant when $P < 0.05$.

587

588 **Table 3. List of metabolites identified in plasma by GC-MS that were significantly**
589 **different between control and gestational diabetes women at different times of**
590 **pregnancy and postpartum.**

Identified compounds	Statictical significance	GDM vs. C (% of change)	RSD in QC (%)
Second trimester of gestation			
Glycerol	VIP/ JK/*	29	12
2-hydroxybutyrate	VIP/ JK/*	51	12
3-hydroxybutyrate	VIP/ JK/*	81	17
Linoleic acid	VIP/ JK/*	25	17
Oleic acid	VIP/ JK	21	9
Palmitoleic acid	VIP/ JK/*	37	14
Palmitic acid	VIP/ JK	13	7
Third trimester of gestation			
Glycerol	VIP/ JK	17	12
Palmitic acid	VIP	13	7
Oleic acid	VIP	13	9
1 month postpartum			
Lactic acid	VIP/ JK	22	11
Proline	VIP	33	26
3 months postpartum			
2-hydroxybutyrate	VIP/ JK	37	12
3-hydroxybutyrate	VIP	128	17
2-ketoisocaproic acid	VIP/ JK	-22	16

Gluconic acid	VIP/ JK	25	21
Palmitic acid	VIP	10	7
Linoleic acid	VIP	19	17
Oleic acid	VIP	15	9
Glycerol	VIP/ JK	13	12

Percent (%) change represents the increase (+) or decrease (−) of the mean in the gestational diabetes group with respect to the control group, the sign indicates the direction of the change. When necessary data were transformed by applying a log(base 2) in order to approximate a normal distribution. Statistical significance reported as the value of multivariate analysis from Variable Importance in Projection (VIP); VIP > 1.0 cutoff was applied. Jack–Knife multivariate statistical analysis (JK) with confidence intervals estimative, 95% confidence level.* data statistically significant according to univariate analysis where $P < 0.05$ was considered significant.

Table 4. List of metabolites identified in plasma by GC-MS that are significantly different in the second trimester of pregnancy and 3 months postpartum between GDM women that were diagnosed T2DM within two years after delivery (GDMT2DM) and those with normoglycemia (GDM-C).

Identified compounds	Statictical significance	GDM-T2DM vs. GDM-C (% of change)	RSD in QC (%)
GDM: Second trimester of gestation			
2-hydroxybutyrate	VIP/ JK/*	94	12
3-hydroxybutyrate	VIP/ JK/*	249	17
Stearic acid	VIP/ JK/*	34	14
Palmitic acid	VIP/ JK	34	7
Palmitoleic acid	VIP/ JK	40	14
Oleic acid	VIP/ JK	38	9
Linoleic acid	VIP/ JK	19	17
Lactic acid	VIP/ JK	-41	11
Threitol	VIP/ JK	-23	18
Sorbitol	JK/*	49	11
GDM: 3 months postpartum			
2-hydroxybutyrate	VIP/ JK/*	161	12
3-hydroxybutyrate	VIP/ JK/*	1511	17
Palmitic acid	VIP/ JK/*	96	7
Palmitoleic acid	VIP/ JK	160	14
Oleic acid	VIP/ JK/*	177	9
Stearic acid	VIP/ JK/*	49	14

Linoleic acid	VIP/ JK/*	125	17
Pyroglutamic acid	VIP/ JK	-25	17
Citric acid	VIP/ JK	-33	26
Lauric acid	VIP/ JK/*	71	26
Hydroxymalonic acid	*	49	26
Lactose	VIP/ JK	-71	15
Serine	VIP/ JK/*	-36	21
Threonine	VIP/ JK/*	-35	20
Glycerol	VIP/ JK	79	12

603 Percent (%) change represents the increase (+) or decrease (−) of the mean in the GDM
604 T2DM group with respect to the GDM-control group (GDM-C). When necessary, data
605 were transformed by applying a log(base 2) in order to approximate a normal distribution.
606 Statistical significance reported as the value of multivariate analysis from Variable
607 Importance in Projection (VIP); a VIP > 1.0 cutoff was applied. Jack–Knife multivariate
608 statistical analysis (JK) with confidence intervals estimative, 95% confidence level. * data
609 statistically significant according to univariate analysis where P <0.05 was considered
610 significant.

FIGURE LEGENDS

Figure 1. Multivariate PLS-DA plots of plasma metabolomic profiles at various times during and after pregnancy for control (●) and GDM women (▲). A. Second trimester of gestation (R2=0.68, Q2=0.13). **B.** Third trimester of gestation (R2=0.69, Q2=-0.11). **C.** One month after delivery (R2=0.61, Q2=-0.21). **D.** Three months after delivery (R2=0.38, Q2=-0.21).

Figure 2. Multivariate PLS-DA plots of plasma metabolomic profiles in the second trimester of gestation and 3 months after delivery for GDM women that are normoglycemic after pregnancy (▲) and those with confirmed postpartum diabetes mellitus (▼). A. Three months after delivery (R2=0.87, Q2=0.55). **B.** Second trimester of gestation (R2=0.88, Q2=0.01).

Figure 3. ROC curves of selected metabolites in the second trimester of gestation, including GDM women that are normoglycemic or those with confirmed postpartum diabetes mellitus. For each metabolite sensitivity is plotted against 100-specificity. **A.** 2-hydroxybutyrate (AUC: 0.90, p= 0.016); **B.** 3-hydroxybutyrate (AUC: 0.867, p= 0.027); **C.** stearic acid (AUC: 0.90, p= 0.016); **D.** sorbitol (AUC: 0.83, p= 0.046).