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Chorell, Elin; Hall, Ulrika Andersson; Gustavsson, Carolina; Berntorp, Kerstin; Puhkala, Jatta; Luoto, Riitta; Olsson, Tommy; Holmäng, Agneta

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Pregnancy to Postpartum Transition of Serum Metabolites in Women with Gestational Diabetes

Elin Chorell,[a*] *Ulrika Andersson Hall,*[b] *Carolina Gustavsson,*[b] *Kerstin Berntorp,*[c,d] *Jatta Puhkala,*[e,f] *Riitta Luoto,*[e,f] *Tommy Olsson,*[a] *and Agneta Holmäng*[b]

[a]*Department of Public Health and Clinical Medicine, Umeå University*

[b]*Institute of Neuroscience and Physiology, University of Gothenburg*

[c]*Department of Clinical Sciences Malmö, Lund University, Malmö, Sweden*

[d]*Department of Endocrinology, Skåne University Hospital, Malmö, Sweden*

[e]*UKK Institute for Health Promotion Research, Tampere, Finland,*

[f]*National Institute for Health and Welfare, Helsinki, Finland*

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[*]*Corresponding author: Elin Chorell, PhD,*

6M, Norrlands universitetssjukhus, 5076, Umeå Universitet, 901 87 Umeå, Sweden

Phone: +46-90-7851326, Fax: +46-90-130760 E-mail: elin.chorell@umu.se

Abstract

Context: Gestational diabetes is commonly linked to development of type 2 diabetes mellitus (T2DM). There is a need to characterize metabolic changes associated with gestational diabetes in order to find novel biomarkers for T2DM.

Objective: To find potential pathophysiological mechanisms and markers for progression from gestational diabetes mellitus to T2DM by studying the metabolic transition from pregnancy to postpartum.

Design: The metabolic transition profile from pregnancy to postpartum was characterized in 56 women by mass spectrometry–based metabolomics; 11 women had gestational diabetes mellitus, 24 had normal glucose tolerance, and 21 were normoglycaemic but at increased risk for gestational diabetes mellitus. Fasting serum samples collected during trimester 3 (gestational week 32±0.6) and postpartum (10.5±0.4 months) were compared in diagnosis-specific multivariate models (orthogonal partial least squares analysis). Clinical measurements (e.g., insulin, glucose, lipid levels) were compared and models of insulin sensitivity and resistance were calculated for the same time period.

Results: Women with gestational diabetes had significantly increased postpartum levels of the branched-chain amino acids (BCAAs) leucine, isoleucine, and valine, and their circulating lipids did not return to normal levels after pregnancy. The increase in BCAAs occurred postpartum since the BCAAs did not differ during pregnancy, as compared to normoglycemic women.

Conclusions: Postpartum levels of specific BCAAs, notably valine, are related to gestational diabetes during pregnancy.

Keywords: Gestational diabetes mellitus, type 2 diabetes mellitus, metabolomics, multivariate statistics, branched-chain amino acids, insulin resistance

1. Introduction

Pregnancy is characterized by extensive metabolic alterations in carbohydrate, fat and protein metabolism to ensure adequate fetal growth and to meet the increased physiological demands of pregnancy, including the additional energy stores required for labor and lactation.

Maternal glycemic control depends on the balance between pancreatic β-cell secretion of insulin, insulin clearance, and insulin action in liver, muscle and adipose tissue [1, 2]. Insulin sensitivity changes considerably during pregnancy and declines progressively in late gestation [1, 3]. The fetoplacental unit has been implicated as a major source of maternal insulin resistance, which is rapidly reversed upon delivery [4]. Inadequate β-cell responsiveness add to the increased insulin resistance and leads to gestational diabetes mellitus which is associated with risk of type 2 diabetes mellitus (T2DM) [3, 5].

Several risk factors correlate highly with gestational diabetes, including advanced maternal age, fetal macrosomia in a previous pregnancy, obesity, and a family history of diabetes [6]. However, early pregnancy screening to identify women at risk for gestational diabetes [2, 7] or postpartum T2DM [3, 5] has not been successful.

Metabolomics studies—comprehensive analysis of low-molecular-weight metabolites—have shown great promise in identifying novel pathways and early biomarkers of insulin resistance and T2DM [8, 9]. Several putative metabolic markers and pathways associated with insulin resistance and T2DM have been identified and validated, such as increased levels of branched-chain amino acids (BCAA) and related metabolites [10, 11]. Only a few studies have examined the metabolomics of hyperglycemia or gestational diabetes during pregnancy; however, the findings suggest that T2DM and gestational diabetes share similar features and that their metabolic signatures might partly overlap [12-15]. Thus, metabolomics may be

useful for identifying biomarkers and understanding the mechanistic underpinnings of gestational diabetes and increased risk for postpartum T2DM.

No study has to our knowledge investigated the unique metabolic transition from a pregnant to a postpartum state and how it differs in women with normal glucose tolerance, women with risk factors for gestational diabetes who remain normoglycemic during pregnancy and women diagnosed with gestational diabetes. We hypothesized that women with gestational diabetes have a unique metabolic profile during the metabolic transition after pregnancy that might help explain pathophysiological mechanisms and potential biomarkers for their elevated risk of postpartum T2DM.

2. Material and Methods

2.1. Sample Collection

To study the postpartum metabolic transition, we included subjects that were sampled both during their third trimester (gestational week 32 ± 4) and postpartum (11 ± 3 months postpartum). Eleven women had gestational diabetes mellitus (GDM group), 24 had normal glucose tolerance (NGT group) and 21 were normoglycemic but at increased risk of GDM (NGT risk group) (Figure 1). The distribution of risk factors for GDM in the three groups is shown in Table 1.

For the GDM group, we recruited pregnant women diagnosed with GDM at Sahlgrenska University Hospital, Gothenburg, Sweden according to the 1991 criteria of the European Association for the Study of Diabetes [16]: oral glucose tolerance test 2-hour plasma glucose ≥10.0 mmol/l. Capillary blood was analyzed with a HemoCue Glucose+ Analyzer (HemoCue, momoCue, Sweden), and blood glucose concentrations were converted to equivalent plasma glucose concentrations [17]. These women were diagnosed at gestational week 26 ± 6 with an oral glucose tolerance test that showed 2-hour plasma glucose 10.9 ± 0.7 mmol/l. After diagnose they were treated to reach normoglycaemia.

Women in the NGT-risk group were recruited at primary health care maternity clinics in the Pirkanmaa region, Finland [18]. Eligible women had at least one of the following risk factors at 8–12 weeks' gestation: body mass index (BMI) ≥25 kg/m^2, GDM or any signs of impaired glucose tolerance or a macrosomic newborn (≥4500 g) in any earlier pregnancy, type 1 or 2 diabetes in first or second-degree relatives, or age ≥40 years. Exclusion criteria were an abnormal oral glucose tolerance test at baseline and type 1 or T2DM before pregnancy, use of neuroleptic drugs, and smoking.

The NGT group consisted of healthy, normoglycemic pregnant women of normal weight from the Gothenburg area, recruited through advertising at the local maternity wards.

All women underwent clinical evaluations during their third trimester (gestational week 32 ± 4) and postpartum (11 ± 3 months). Fasting blood samples were collected at each visit and analyzed for glucose, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), insulin, and free fatty acids (FFA). Samples from the GDM and NGT groups were analyzed at the accredited Clinical Chemistry Laboratory, Sahlgrenska University Hospital (SWEDAC ISO 15189). Samples from the NGT-risk group were analyzed at the UKK Institute for Health Promotion Research, Tampere (glucose, cholesterol, HDL) or the MCA Research Laboratory, Turku, Finland (LDL, insulin, FFA). An aliquot of EDTA plasma from all samples was frozen and stored at –80° C for metabolomics analysis. For analysis of insulin resistance, fasting insulin, glucose, and FFA levels were used to calculate the homeostatic model assessment (HOMA) [19] and insulin sensitivity, revised quantitative insulin sensitivity check index (revised QUICKI) [20].

All participants received oral and written information on the study and gave written consent to participate. The studies were approved by the Regional Ethical Review Board, University of Gothenburg. (Dnr 402-08) and of Pirkanmaa Hospital District (Reference number R06230, 19.1.2007).

2.2. Sample Preparation and Metabolomics Analysis

The run order and sample preparation were designed to minimize biases from sample collection site, sample preparation, and analysis that could confound the interpretation of the results. Samples from the same participant were prepared and analysed in close connection whilst keeping the internal sample order randomized. In total, 112 samples and 33 quality-control samples were analyzed by gas chromatography–time-of-flight mass spectrometry

(GC-TOF/MS). Quality control samples, pooled from all included samples, were continuously analyzed. Before GC-TOF/MS analysis, serum metabolites were extracted with MeOH-H_2O by a two-step derivatization procedure [21]. The samples were then injected into an Agilent 6890 gas chromatograph equipped with a 10-m fused silica capillary column (inner diameter, 0.18 mm) with a chemically bonded 0.18-µm DB 5-MS stationary phase (J&W Scientific, Folsom, CA). The column effluent was introduced into the ion source of a Pegasus III TOF/MS, GC-TOF/MS (Leco, St Joseph, MI). Drift removal and data normalization are described in the supplemental data.

2.3. Data Processing

To quantify and identify metabolites, we used an in-house MATLAB script. Putative metabolites were extracted by using unique mass channels and retention indices matched to our in-house mass spectral library at the Swedish Metabolomics Centre (www.swedishmetabolomicscentre.se). The data set was filtered to remove double peaks and noisy spectra, and only unique spectral profiles with a relative standard deviation (RSD) <40%, calculated from quality control samples, were included in sample comparison modeling. Criteria set by the Human Metabolome Database (www.HMDB.ca) were used to assign extracted components to different compound classes (amino acids and derivatives, BCAA, carbohydrates, lipids, or no class).

2.4. Statistical Analysis

Groupings, outliers, and trends were detected by principal components analysis (PCA). For each subject, the postpartum sample was subtracted from sample collected during pregnancy; missing data were excluded. Next, a variant of orthogonal partial least squares (OPLS) (OPLS) [22], OPLS-effect projections [23], was used to extract relevant metabolic profiles of the pregnancy to postpartum transition, based on paired analyses of the individual effects (i.e., the effect of the postpartum transition). Since each subject served as her own control, this

strategy minimizes the influence of instrumental drift, site differences, and interindividual variation [23].

To validate the multivariate models, *P* values for the differences between the predefined classes were calculated by analysis of variance (ANOVA) based on the cross-validated OPLS scores (CV-ANOVA); $P < 0.05$ was considered significant. Special consideration was taken to ensure proper cross-validation groups (i.e., that the same participant/replicate was kept in the same group) to reduce the risk of creating overfitted models. A metabolite was considered to contribute significantly to the metabolite profile if it was significantly altered according to the multivariate confidence interval, based on jack-knifing [24], and a significant univariate *P*-value, both on a 95% significance level. Univariate *P* values were calculated with the *t* test (sample size >20) or the Wilcoxon signed-rank test (sample size <20).

3. Results

3.1. Clinical Measurements

Clinical characteristics and measurements in the three cohorts during and after pregnancy are shown in Table 2. BMI during pregnancy was higher in the NGT-risk and GDM groups than in the NGT group ($P < 0.01$). Gestational weight gain was lowest in the GDM group ($P < 0.05$ versus NGT-risk). Insulin resistance (HOMA-IR) was higher in the NGT-risk group than in the NGT group ($P < 0.01$), and insulin sensitivity (revised QUICKI) was lowest in the GDM group ($P < 0.05$ vs NGT). Postpartum, the NGT-risk and GDM groups still had significantly higher BMIs and higher waist-to-height ratios than the NGT group.

The postpartum shift in clinical measurements is shown in Figure 2. In the NGT group, postpartum plasma glucose and revised QUICKI increased significantly, and HOMA and insulin levels decreased, indicating normalization of metabolic status (Figure 2). The NGT-risk group also increased their postpartum plasma glucose, HOMA, and insulin, indicating normalization of blood glucose, but their insulin sensitivity decreased along with cholesterol. In the GDM group, postpartum glucose and revised QUICKI increased, indicating normalization of insulin sensitivity, but blood cholesterol and LDL were not lowered to the same extent as in the NGT group.

The use of dietary supplements in the different groups is found in table S2. It shows no differences in use of supplements between the different groups during pregnancy or postpartum.

3.2. Postpartum Plasma Metabolic Profiles

Initial inspection of the metabolic profiles by principal component analysis (PCA) did not reveal outliers in samples collected during pregnancy or postpartum. The largest systematic variations were related to diagnosis and sample collection site (i.e., the NGT -risk group was separated from the GDM and NGT groups in the first PC) (Figure S1). Since the sampling

was longitudinal, we focused on the postpartum metabolic transition profiles for the different diagnosis groups to circumvent differences in site from confounding of interpretation of the results. The postpartum metabolic profile of 66 identified putative metabolites is shown for NGT-risk and GDM groups in Supplemental Table S1.

The postpartum metabolic transition models (OPLS-EP) were based on the difference between the pregnancy and postpartum values for each subject. Diagnosis-specific OPLS models (CV-ANOVA $P > 0.001$), which describe the metabolic profile of a postpartum transition, were significantly different for the NGT-risk and GDM groups (Figure 3) but not for the NGT group. Therefore, all findings related to the NGT group are from univariate analysis of single putative metabolites (Table S1). The predictability of the OPLS models (i.e., the percent of the total variation predicted by the calculated latent variable/OPLS component, Q2) was >75%, and two significant components, one predictive and one orthogonal, were extracted for each model. Only the predictive component (the systematic variation related to the postpartum transition) is shown in Figure 2.

The postpartum metabolic transition profiles differed in the GDM and NGT-risk groups. In the GDM group, the BCAAs, tryptophan, ornithine, proline, lactose, and a number of hexoses increased significantly postpartum, while glutamic acid and cholesterol decreased significantly. Notably, among the BCAAs valine levels differed most between the study groups and also showed the most pronounced difference between samples collected during pregnancy and postpartum (Figure 4). Indeed, BCAAs did not differ between the NGT and GDM groups (collected at the same site) during pregnancy ($P > 0.92$), but all BCAAs differed significantly ($P < 0.02$) postpartum (Figure 4).

Postpartum, asymmetric dimethylarginine and citrulline levels increased significantly in the GDM and NGT-risk groups but not in the NGT group ($P > 0.27$), and the level of polyunsaturated docosahexaeonic acid (DHA, 22:6n-3) decreased significantly in the GDM and NGT groups but not in the NGT-risk group. Also significantly reduced ($P < 0.03$) in the

NGT group were postpartum levels of palmitic acid (16:0) and three unsaturated 18C fatty acids, namely linoleic acid (18:2), elaidic acid (18:1, trans), and oleic acid (18:1, cis). In all women, threonine and allothreonine levels decreased and the ketoleucine level increased postpartum. The postpartum transition of all putative metabolites is shown in Table S1.

4. Discussion

This study shows that women with GDM have a substantially different metabolic profile during the pregnancy to postpartum transition than women with NGT, including those at increased risk for GDM. Postpartum, the GDM group had a significant increase in the BCAAs (leucine, isoleucine, and valine) and a less pronounced normalization of circulating lipids. The increase was related to higher postpartum BCAA levels in the GDM group, since BCAAs did not differ between the GDM and the NGT group during pregnancy, in line with earlier studies [25, 26]. Postprandial BCAAs 6 weeks postpartum are also higher in insulin-treated women with GDM women than in NGT women [27]. These alterations in protein and lipid metabolism may point to pathophysiological mechanisms and potential biomarkers to predict the development of T2D, after GDM.

Pregnancy entails an increased demand for energy, including amino acids, to enable the fetus and placenta to grow. Thus, normal pregnancy induces hypoaminoacidemia, which reduces BCAAs in the circulation, potentially to conserve nitrogen and increase protein synthesis aimed at conservation and accretion of nitrogen by the woman and the fetus [28]. This can explain the conflicting reports on BCAA levels during pregnancy [25, 26, 29]. Lindsay et al showed a decrease in two BCAAs during normal pregnancy, i.e. leucine and valine, suggesting that the amino acids should increase postnatally although no study before have investigated this transition [30]. We could not detect a significant postpartum increase in any of the BCAAs, in NGT or NGT risk groups. However we found a non-significant increase in BCAAs in the NGT group (data not shown). This might indicate that this study was too small to detect the increment back to prepregnancy levels postpartum. Another possibility is that postpartum normalization of BCAA among NGT individuals requires more time than 6-12 months.

In women with postpartum GDM, increased levels of BCAAs, or other mitotoxic/lipotoxic metabolites from these amino acids, might increase risk for T2DM through their negative effects on β-cell function [31]. Insulin resistance can also be influenced by BCAA metabolites. 3-hydroxyisobutyrate (3-HIB), a catabolic intermediate of the BCAA valine, secreted from muscle cells, activates endothelial fatty acid transport, stimulates muscle fatty acid uptake in vivo, and promotes lipid accumulation in muscle, leading to insulin resistance [32]. 3- Hydroxyisobutyrate levels were higher in muscle from both *db/db* mice and humans with diabetes than in those without. The elevated valine levels in the GDM group can thus contribute to decreased insulin signaling and worsen insulin resistance. However, we could not find any significant postpartum alteration in 3-hydroxyisobutyrate (Table S1).

We also found significant postpartum alterations in several other interesting amino acids. For example, alanine and arginine levels were increased postpartum in both the NGT-risk and the GDM groups, while leucine and proline were increased only in the GDM group. These amino acids stimulate insulin secretion and could thereby contribute to exhaustion of β-cells by causing endoplasmic reticulum stress [10, 33-35]. We also found increased levels of citrulline in the GDM and NGT-risk groups and of ornithine in the GDM group. Citrulline and ornithine concentrations increase in mice with diet-induced obesity associated with hyperglycemia, hyperinsulinemia, and nonalcoholic fatty liver disease [36]. Chronic elevation of these potential β-cell secretagogues might lead to loss of insulin secretion if inherited abnormalities of beta cell function or mass predispose to the development of diabetes. Insulin sensitivity (revised QUICKI) increased in absolute terms in both the NGT and GDM groups postpartum and was significantly lower in the GDM group and decreased in the NGT-risk group. Concomitantly, insulin resistance (HOMA-IR) decreased postpartum in the NGT and the GDM groups but increased in the NGT-risk group. Notably, the revised QUICKI includes free fatty acid in modeling insulin sensitivity, resulting in a better correlation with

the clamp-based index of insulin sensitivity and greater discriminatory power in cases of mild insulin resistance [37, 38].

The lack of a significant postpartum metabolic transition profile in the NGT group suggests that the metabolic shift is less pronounced in this group. Nevertheless certain lipid species decreased postpartum, suggesting normalization of lipid levels. Specifically, cholesterol, LDL, HDL, palmitic acid, three unsaturated 18C fatty acids, and DHA decreased. Similarly, in the NGT-risk group, cholesterol, LDL, and HDL decreased during the postpartum transition, but the fatty acids remained unchanged. In the GDM group, however, only postpartum cholesterol and DHA levels decreased. In line with this, we found several circulating lipids (LDL, HDL, cholesterol) that were significantly higher in women with GDM as compared to those with normal glucose tolerance during pregnancy. Elevated circulating lipids during late pregnancy, partly due to rising blood levels of lipolytic placental hormones, may be key for the increase in insulin resistance [39]. Chronic exposure of islets to elevated concentrations of fatty acids can also impair glucose-stimulated insulin secretion [40, 41].

A large body of evidence implicates lipids, BCAA and other amino acids in the development of tissue disorders, metabolic disease and insulin resistance. These findings suggest that these abnormalities are driven by the combined effects of lipids and BCAA or other amino acids. In addition, there might be interactions of excess BCAAs and lipids in the development of β-cell impairment. The metabolic basis for gradual dysregulation of glucose-stimulated insulin secretion in T2DM is not completely understood, in part because both lipids and amino acids have complex and similar effects on β-cells. Fatty acids can serve as amino acids or secretagogues and increase insulin secretion through a combination of messengers produced during metabolism and through activation of cell-surface G protein-coupled receptors [42, 43]. In this way, chronic exposure of islets to elevated concentrations of fatty acids impairs

glucose-stimulated insulin secretion. The chain length, degree of unsaturation, and the spatial configuration of fatty acids influence their effects on β-cell function [44].

A limitation of this study is the size of the GDM group. Importantly, this was considered in the multivariate analysis, in which each woman served as her own control during extraction of metabolic profiles. This strategy potentially increases statistical power by reducing site- and intra-individual biases that could confound the interpretation of the results. Also, nutritional and physical activity patterns are important factors that might influence the metabolic pattern and should be taken in consideration in future studies.

In conclusion, our findings, especially the validity of BCAAs and lipids as potential pathophysiological factors explaining the development to T2DM, negatively affecting β-cell function and insulin sensitivity, need to be further validated in combination with clinical follow-up data on the actual development of T2DM. The ultimate goal is to develop clinical easy-to-use, widely applicable markers to prevent T2DM after GDM in at-risk-women.

6. Acknowledgements and contribution statement

E.C performed the metabolomics analysis and the multivariate statistics, wrote the manuscript and is the guarantor of this work. U.A.H analyzed clinical data and contributed to writing of manuscript, C.G collected and compiled clinical data. K.B reviewed and edited the manuscript and contributed to discussion. J.P compiled clinical data R.L collected clinical samples and contributed to the discussion. T.O wrote the manuscript and contributed to discussion. A.H designed the study, wrote the manuscript, collected clinical samples and is the guarantor of this work. We thank Stephen Ordway for editorial assistance.

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438 **Figure Legends**

439 **Figure 1 – Study protocol.** Flow chart illustrating included women that were either
440 normoglycemic (NGT), normoglycemic with increased risk for developing gestational
441 diabetes (NGT risk) or diagnosed with gestational diabetes (GDM) and time table for
442 sampling.

443 **Figure 2—Postpartum shift in clinical measurements.** Absolute changes in concentration
444 from the third trimester (gestational week 32±0.6) to postpartum (10.5±0.4 months) for
445 clinical measurements and mathematical models of insulin resistance (HOMA-IR) and insulin
446 sensitivity (revised QUICKI). Values are mean ± SD. *$P < 0.05$ postpartum versus late
447 pregnancy (paired *t*test. #$P < 0.05$ (one-way ANOVA and Tukey posthoc test).

448 **Figure 3—Multivariate analysis.** Diagnosis-specific OPLS models displaying the metabolic
449 profile of the postpartum transition (OPLS model weights, w*[1]), i.e. the significantly altered
450 plasma metabolites when comparing samples collected during the third trimester (gestational
451 week 32±0.6) to those collected postpartum (10.5±0.4 months). (*A)* NGT -risk group. (*B*)
452 GDM group. No significant model was obtained for the NGT group. Plasma components with
453 positive axis values were higher postpartum and those with a negative axis values were lower
454 than during pregnancy. Only components that were altered significantly postpartum are
455 shown (significant by the OPLS multivariate 95% confidence interval (based on jack-knifing)
456 and univariate $P < 0.05$ (paired *t* test).

457 **Figure 4—Branched-Chain Amino Acids.** Relative concentrations of the branched-chain
458 amino acids (BCAA, valine, leucine and isoleucine), detected by GC/MS- based
459 metabolomics. All BCAAs were higher postpartum (black dots) in the gestational diabetes
460 mellitus (GDM) group, $P < 0.01$) than during pregnancy (white dots). No significant
461 postpartum alterations were detected in the normal glucose tolerance (NGT) or NGT-risk
462 groups. All three BCAAs differed between NGT and GDM group postpartum ($P < 0.02$) and

valine and isoleucine differed between GDM and NGT-risk postpartum; no difference were seen during pregnancy. The unique mass channel for each amino acid used for quantification is stated on each *y*-axis. Red line indicates mean values and the grey box represent 95% standard deviation of the sampling distribution.