Fetal Exposure to Altered Amniotic Fluid Glucose, Insulin, and Insulin-Like Growth Factor–Binding Protein 1 Occurs Before Screening for Gestational Diabetes Mellitus

DANIEL K. TISI, MSC[1]
DAVID H. BURNS, PHD[2,3]
GARY W. LUSKEY, MD[3,4]
KRISTINE G. KOSKI, PHD[1,3]

OBJECTIVE — We explored the possibility that perturbations in amniotic fluid glucose, insulin, and insulin-like growth factor–binding protein 1(IGFBP1) and/or metabolic acids exist before routine screening for GDM.

RESEARCH DESIGN AND METHODS — We selected consenting mother-infant pairs ($n = 408$) who met our inclusion criteria (singleton pregnancy, no genetic abnormalities, and no preexisting diabetes) and for whom sufficient amniotic fluid and appropriate medical information were available. We compared birth outcomes and second trimester amniotic fluid glucose, insulin, IGFBP1 concentrations, and amniotic fluid lactic, β-hydroxybutyric, and uric acids of mothers with gestational diabetes mellitus (GDM) ($n = 52$) with those of mothers with no diagnosis of GDM at >24 weeks ($n = 356$).

RESULTS — Higher amniotic fluid glucose, lactic acid, uric acid, and insulin and lower IGFBP1 concentrations were present by 15.1 ± 0.1 weeks in mothers in whom GDM was subsequently diagnosed. However, logistic regression showed that second trimester amniotic fluid glucose, but not insulin, IGFBP1, or metabolic acids was associated with an increased odds ratio (1.2 [95% CI 1.052–1.338]) for diagnosis of GDM at 24–28 weeks. In addition, probability contour maps that accounted for nonlinear relationships among the dynamically changing amniotic fluid constituents showed an increased risk for GDM with elevated second trimester amniotic fluid glucose in combination with either elevated amniotic fluid insulin or low amniotic fluid IGFBP1

CONCLUSIONS — Fetuses are exposed to increased amniotic fluid glucose before 15 weeks of gestation, suggesting that metabolic perturbations are underway before diagnosis and that earlier screening and intervention may be warranted.

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Gestational diabetes mellitus (GDM) is defined as a state of hyperglycemia arising during gestation that leads to increased glucose delivery to the developing fetus. Glucose is considered to be the principal metabolic fuel and supplies 50–80% of fetal glucose needs (1) with amniotic fluid supplying another 10–15% via fetal swallowing (2). Reportedly, amniotic fluid glucose concentrations are higher in mothers with type 1 or type 2 diabetes than in those without diabetes (3) and in those with GDM than in those without GDM (4). Amniotic fluid insulin is also elevated in mothers with GDM versus those without GDM (4–6). Some have suggested that amniotic fluid insulin may be a more sensitive early predictor of GDM than amniotic fluid glucose, but neither has emerged as an early screening tool (4). More recently, perturbations in insulin-like growth factors and their binding proteins have been implicated in diabetic pregnancies (7). In particular, lower concentrations of insulin-like growth factor–binding protein 1 (IGFBP1) in maternal plasma during the second trimester before diagnosis of GDM (8) and an inverse relationship with cord blood IGFBP1 of mothers with GDM at term and birth weight (9) have been described.

It is well known that GDM is associated with poor perinatal outcomes, with macrosomia, and with a higher prevalence of diabetes in the offspring of mothers with GDM (10,11), but how soon during development these fetal insults begin is not clear. Two recent studies have linked subsequent diagnosis of GDM with irreversible oxidative damage to amniotic fluid albumin (12) and to lacticacidemia and fetal hypoxia in cord blood at term even in mothers with well-controlled GDM (13). Mothers with GDM also show evidence of altered ketogenesis and higher circulating concentrations of β-hydroxybutyrate that may be linked to increased gluconeogenesis and not to β-oxidation (14) and to increased uric acid (15) that could be related to hypoxia that often occurs with diabetes.

GDM is usually diagnosed between 24 and 28 weeks of gestation by an oral glucose tolerance test (OGTT) (16). In this study, we explore the possibility that early fetal exposure to perturbations in second trimester amniotic fluid glucose, insulin, and IGFBP 1 and/or amniotic fluid lactic, β-hydroxybutyric, and uric acids may exist before current routine screening for GDM.

RESEARCH DESIGN AND METHODS

— From 2000 to 2004, pregnant women undergoing age-related amniocentesis for genetic testing at St Mary's Hospital Center (Montreal, QC, Canada) were approached to participate in this study. Signed consents allowed researchers to collect frozen amniotic fluid samples and to review medical records after genetic testing. Application of exclusion criteria (multiple births, genetic

From the [1]School of Dietetics and Human Nutrition, McGill University, Montreal, Quebec, Canada; the [2]Department of Chemistry, McGill University, Montreal, Quebec, Canada; the [3]Faculty of Medicine, McGill University, Montreal, Quebec, Canada; and the [4]Division of Perinatal/Fetal Medicine, St Mary's Hospital Centre, Montreal, Quebec, Canada.
Corresponding author: Kristine G. Koski, kris.koski@mcgill.ca.
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anomalies, and preexisting type 1 or type 2 diabetes) resulted in 408 mother-infant pairs having birth weights, gestational age, and sex recorded in the maternal chart and for whom the screening for and diagnosis of GDM using a 1-h 50-g OGTT at 24–28 weeks of pregnancy was performed with a cutoff of 10.3 mmol/l for diagnosis of GDM, which was in place at the time of our recruitment. At present a value of >10.3 mmol/l is still used to diagnose GDM (16). Ethics approval was obtained from McGill University and St Mary's Hospital Centre.

Prepregnancy weight and height, age, smoking status (1 = yes and 0 = never or quit before pregnancy), parity, infant sex, birth weight, and gestational age were verified from medical charts. Gestational age at both the time of amniocentesis and at parturition was calculated based on physicians' estimates using last menstrual period. Ethnicity was classified as Caucasian, Asian, or other. Maternal prepregnancy BMI was categorized into four groups: <18.5 kg/m^2 for underweight, 18.5–24.9 kg/m^2 for normal, 24.9–29.9 kg/m^2 for overweight, and ≥30 kg/m^2 for obese.

Biochemical analyses

Frozen amniotic fluid samples (−80°C) were analyzed for glucose, insulin, and IGFBP1. Insulin was analyzed using the Beckman Access ultrasensitive assay system (Beckman Coulter, Brea, CA), a one-step immunoenzymatic assay that adds a monoclonal anti-insulin conjugate, antibody-coated paramagnetic particles, and a chemiluminescent substrate to the reaction vessel. This new, more specific, assay measures insulin to within 0.03–300 μIU/l. Glucose was analyzed after adaptation of a hexokinase assay kit (No. 6082; Abbott Laboratories, North Chicago, IL) for use with a microplate reader. Total IGFBP1 was measured by ELISA (DSL kit 10-7800; Diagnostics Systems Laboratories, Webster, TX).

The three metabolic acids were measured as follows: β-hydroxybutyric acid by procedure 310-UV (reagent 310-3, β-hydroxybutyric dehydrogenase solution 310-4, and β-hydroxybutyric acid calibrator solution 310-50; Sigma Aldrich Canada, Oakville, ON, Canada); uric acid by the A-gent chemistries (reagent 6184, standards 6008-02, procedure 69-0163; Abbott Laboratories Diagnostics Division, North Chicago, IL); and lactic acid by procedure 735 (Sigma-Aldrich). All kits were adapted for microplate assay. β-Hydroxybutyric and uric acids were read at 355 nm using a Victor2 plate reader (Wallac; Gaithersburg, MD, a subdivision of PerkinElmer Life and Analytical Sciences, Boston, MA), and lactate was read in a BioTek reader (BioTek Instruments, Winooski, VT) at 540 nm.

Statistical analyses

All data analyses were performed using SAS (version 9.1; SAS, Cary, NC) with $P < 0.05$ set as the minimum for statistical significance. All nonnormally distributed continuous data were transformed using square root (amniotic fluid glucose and gestational age at time of amniocentesis) and logarithmic processing (prepregnancy weight, BMI, and amniotic fluid insulin). We performed ANCOVA, controlling for maternal BMI, age, infant sex, smoking, and parity and where required for amniocentesis week and ethnicity for each of our amniotic fluid constituents if there were significant differences between the populations with and without GDM. Separate logistic regressions for GDM with each amniotic fluid constituent entered individually in the model were performed; factors known to contribute to the emergence of GDM (BMI, maternal age, parity, and ethnicity) (16) were also included in each logistic regression model.

In addition, Bayesian contour maps were created to assess risk for GDM because this approach did not presume a linear relationship and would account for metabolic interrelationships resulting from these dynamically changing amniotic fluid constituents. Each outcome (GDM vs. non-GDM) was separately modeled with Gaussian mixture modeling and an expectation maximization of a log-likelihood procedure that uses a quasi-Newton algorithm written in Matlab V6.5 (Mathworks). The posteriori distributions were plotted as contour maps for which lines of equal height in the joint density function were created using the measured data (17). Specificity, sensitivity, and accuracy for each contour map were evaluated using the maximum posteriori probability for assignment of an outcome. Specificity was calculated as number of true negatives/number of true negatives + number of false positives. Sensitivity was calculated as number of true positives/number of true positives + number of false negatives. Accuracy was calculated as number of true correct predictions/total number of individuals.

RESULTS

A total 408 pregnant mothers were screened for GDM at >24 weeks gestation; 52 (11.3%) met the Canadian Diabetes Association (2008) criteria for GDM with a 1-h 50-g OGTT ≥10.3 mmol/l (16). Otherwise, our multiethnic population (64% Caucasian, 20% Asian, and 16% other) were healthy, nonsmoking (84%) mothers who delivered infants with a mean birth weight of 3,462 ± 22 g at term (39.5 ± 0.07 weeks). Comparisons for insulin and IGFBP1 according to week of pregnancy when amniocentesis was performed (12–23 weeks; 15.2 ± 0.03) showed that amniotic fluid insulin concentrations collected in women whose amniocentesis were done after 16.5 weeks were higher than those in women having amniocentesis performed before 15.5 weeks, and IGFBP1 was higher after 15.5 weeks than at earlier time points. In contrast, amniotic fluid glucose and β-hydroxybutyric, lactate, and uric acid concentrations did not differ across these weeks of gestation (data not presented).

Comparisons between mothers with and without GDM and their offspring are reported in Table 1. Mothers with GDM were older, shorter, and heavier; in fact the proportion of overweight (BMI >26 kg/m^2) in mothers with vs. without GDM was 36 vs. 19% and for obese mothers (BMI >30 kg/m^2) was 21 vs. 7%, respectively ($P < 0.0001$). Of interest, a higher proportion of mothers with GDM were Asian (46 vs. 16% in the non-GDM group; $P < 0.05$). Parity did not differ between mothers with and without GDM ($P = 0.0699$). Offspring of mothers with GDM were heavier (3510 vs. 3445 g; $P < 0.05$) and had a higher sex-corrected birth-weight-for-gestational-age percentile (64.8 ± 3.7 vs. 51.6 ± 1.4%; $P < 0.05$). Markedly more infants of mothers with GDM were born large for gestational age (21 vs. 7%; $P < 0.05$) even though these infants were born earlier than their non-GDM counterparts (39.0 ± 0.21 vs. 39.6 ± 0.07 weeks; $P < 0.05$). Despite these differences, the majority of infants were born appropriate for gestational age in our non-GDM (88.5%) and GDM groups (75.1%). On average, GDM was associated with a 176-g increase in birth weight in a multiple linear regression that controlled for maternal prepregnancy weight and height, smoking behavior, gestational age, parity, and infant sex. This model captured 32% of the variability in infant birth weight.

Mothers with GDM had higher second trimester amniotic fluid glucose, lac-

Table 1—*Maternal, infant, and amniotic fluid characteristics for mother-infant pairs with GDM at >24 weeks vs. those without GDM*

Characteristics	Non-GDM	GDM	*P* value
Maternal			
Height (m)	1.62 ± 0.004	1.60 ± 0.01	0.0136
Prepregnancy weight (kg)	61.9 ± 0.62	67.5 ± 2.9	0.0231
BMI (kg/m^2)	23.4 ± 0.21	26.3 ± 1.0	<0.0001
% overweight (BMI 25–30 kg/m^2)	19	36	
% obese (BMI ≥30 kg/m^2)	7	21	
Parity	1.1 ± 0.05	1.4 ± 0.14	0.0699
Maternal age (years)	37.8 ± 0.13	38.7 ± 0.32	0.0155
Infant			
Gestational age at birth (week)	39.6 ± 0.07	39.0 ± 0.21	0.0062
Birth weight (g)	3,445 ± 23	3,581 ± 77	0.0408
% AGA	88.5	75.1	
% LGA	7	21	
Birth weight for gestational age (% ranking)	51.6 ± 1.4	64.8 ± 3.7	0.0008
Amniotic fluid			
Amniocentesis week	15.1 ± 0.05	15.3 ± 0.16	0.1324
Glucose (mmol/l)*	3.84 ± 0.12	5.61 ± 0.47	<0.0001
Insulin (pmol/l)*	0.57 ± 0.02	0.86 ± 0.13	<0.0001
IGFBP1 (μg/ml)*	35,602 ± 1,578	25,856 ± 3,308	0.0246
Lactic acid (mmol/l)*	7.9 ± 0.1	8.8 ± 0.3	0.0061
β-Hydroxybutyric acid (μmol/l)*	220 ± 6.7	231 ± 21	0.6968
Uric acid (μmol/l)*	173 ± 4.3	204 ± 13	0.0142

Data are reported as means ± SEM. Maternal prepregnancy weight, BMI, gestational week of amniocentesis, and amniotic fluid insulin, glucose, and β-hydroxybutyric acid were either square-root or log transformed to normalize skewness and kurtosis. AGA, appropriate for gestational age; LGA, AGA, large for gestational age. *Covariates for amniotic fluid variables included maternal BMI, smoking, parity, age, ethnicity, and week of amniocentesis. $P < 0.05$.

tic and uric acids, and insulin and lower IGFBP1 (Table 1). Inclusion of one or more of the following covariates, maternal BMI, age, ethnicity, parity, smoking behavior, infant sex, and gestational age at the time of amniocentesis, did not alter these significant relationships for glucose and insulin but did for IGFBP 1. We were able to show that both maternal age and ethnicity explained the difference in amniotic fluid IGFBP1 concentrations between mothers with and without GDM because inclusion of age and ethnicity as covariates eliminated the significant difference between these two groups. When we subdivided our amniotic fluid constituents by ethnicity, we also observed that Asians had higher insulin (0.70 ± 0.05 vs. 0.54 ± 0.02 pmol/l) and lower IGFBP1 (27,949 ± 2,974 vs. 37,472 ± 1,814 μg/ml) than Caucasians; other ethnic groups had intermediate concentrations that did not differ. Amniotic fluid glucose did not differ by ethnicity. Finally multivariate logistic regression revealed that maternal BMI, age, ethnic origin, and increased second trimester amniotic fluid glucose were all associated with increased odds of mothers developing GDM (i.e., 10.3, 16.4, 81.1, and 18.6%, respectively) (Table 2). Neither amniotic fluid insulin (odds ratio 1.679 [95% CI 0.915–3.081]), IGFBP1 (1.00 [1.00–1.00]), nor metabolic acids entered as a significant independent predictor in similar models for GDM.

Because of the metabolic interrelationship of amniotic fluid glucose with both amniotic fluid insulin and amniotic fluid IGFBP1, we explored the possibility that changes in one amniotic fluid constituent could influence the concentration of the other. We presumed that the interrelationships would be nonlinear and therefore used two-dimensional probability plots to explore potential metabolic interrelationships with pairs of amniotic fluid constituents. Two sets of contour maps for risk of GDM were created that paired amniotic fluid glucose with amniotic fluid insulin (Fig. 1*A*) and amniotic fluid glucose with amniotic fluid IGFBP1 (Fig. 1*B*). The contour map combining amniotic fluid glucose with amniotic fluid insulin (Fig. 1*A*) showed that high amniotic fluid glucose (>7.5 mmol/l) in combination with high amniotic fluid insulin (>1.5 pmol/l) was associated with >80% risk of mothers subsequently having a diagnosis of GDM. It also showed that at any glucose concentration, an elevation to 1.5 pmol/l in amniotic fluid insulin increased the likelihood of mothers having a diagnosis of GDM; 4.6% of our mothers who developed GDM met these criteria. The accuracy for our glucose and insulin contour map was 68%, its specificity was 74%, and its sensitivity was 27%. Our second contour map (Fig. 1*B*) comparing amniotic fluid glucose with IGFBP1 showed that higher amniotic fluid glucose (>7.5 mmol/l) in combination with lower concentrations of amniotic fluid IGFBP1 (<3 μg/ml) were associated with a higher risk of mothers subsequently having a diagnosis of GDM. This contour map had an accuracy of 64%, a specificity of 68%, and a sensitivity of 38%. We concluded on the basis of our specificity compared with our sensitivity measurements that we were more likely to "identify" those mothers who would not develop GDM than predict its later emergence.

CONCLUSIONS — Population characteristics and pregnancy outcomes of

Table 2—*Logistic regression for GDM*

Characteristic	Odds ratio (95% CI)
Maternal prepregnancy BMI (kg/m^2)	1.103 (1.034–1.176)
Maternal age (years)	1.164 (1.019–1.330)
Maternal ethnicity	1.811 (1.225–2.679)
Amniotic fluid glucose (mmol/l)	1.186 (1.052–1.338)*
Amniotic fluid insulin (pmol/l)	1.679 (0.915–3.081)*
Amniotic fluid IGFBP1 (μg/ml)	1.00 (1.00–1.00)*
β-Hydroxybutyric acid (μmol/l)	1.00 (0.99–1.001)*

*Amniotic fluid constituents were included one at a time in different GDM logistic regression models. Glucose was the only significant amniotic fluid constituent that predicted a later diagnosis of GDM because the 95% CI did not pass through 1. The model controlled for prepregnancy BMI, maternal age, and ethnicity, where parity and smoking were also entered but were not significant. $n = 386$.

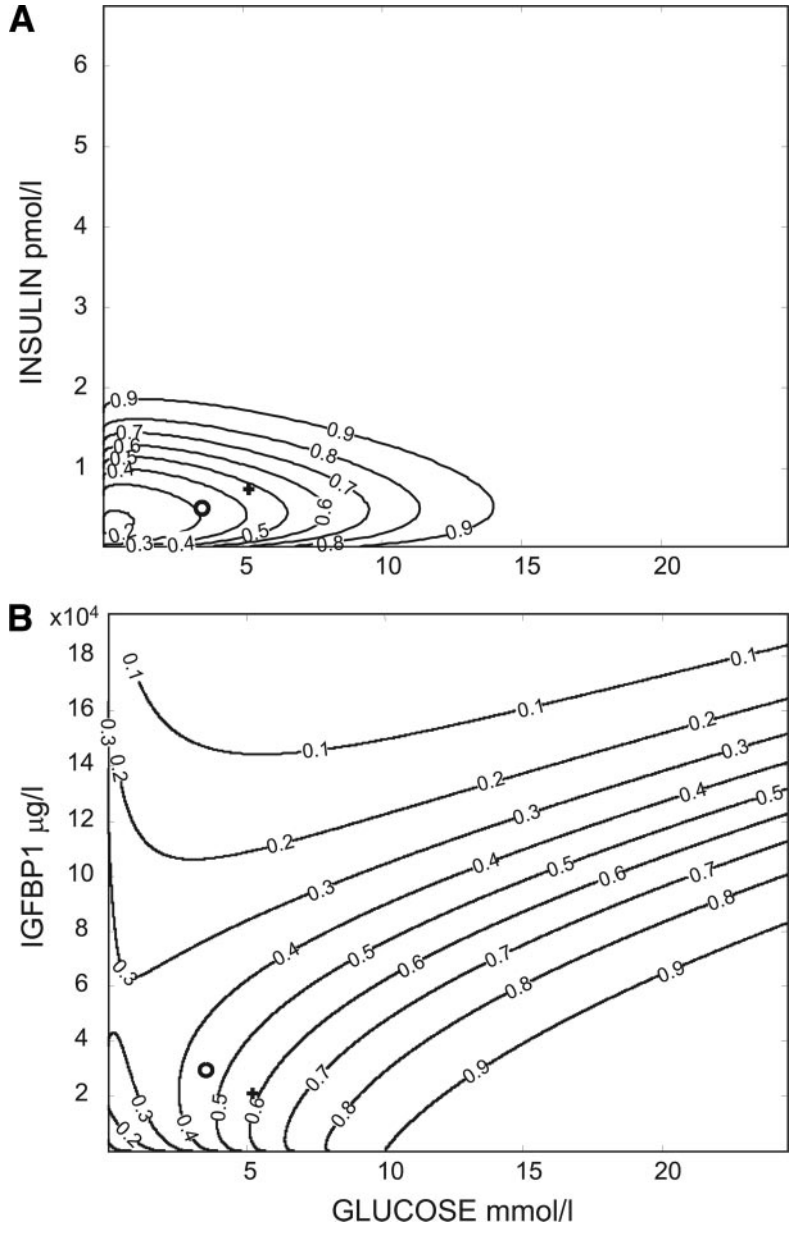

Figure 1—A*: Class-conditional Bayesian a posteriori probability density plotted as a contour map for amniotic fluid glucose paired with amniotic fluid insulin.* B*: Similar class-conditional Bayesian a posteriori probability density contour map for second trimester amniotic fluid glucose and IGFBP1.* ○*, mean for the population without GDM;* +*, mean for the population with GDM.*

our mothers, who were older, were typical of the Canadian population at large. The use of second trimester amniotic fluid glucose and insulin as prognosticators for GDM has previously been investigated in a few studies but with variable results (4,6,18). Our study, with its larger population, resulted in four major findings. We showed that *1*) GDM, which occurred in lean, overweight, and obese women, was associated with a 176-g increase in birth weight in our study population, *2*) amniotic fluid glucose, lactic and uric acids, and insulin were elevated by 15 weeks whereas IGFBP1 was decreased in mothers subsequently having a diagnosis of GDM, *3*) despite perturbations in insulin, IGFBP1, and metabolic acids, only amniotic fluid glucose was associated with an increased odds of mothers developing GDM once all other risk factors were controlled for, and *4*) two-dimensional contour maps that classified amniotic fluid glucose in combination with either amniotic fluid insulin or IGFBP1 were better at "ruling out" GDM than in "predicting" its occurrence. These results underscore the existence of early metabolic perturbations in amniotic fluid glucose that may be related to dynamic changes in insulin and IGFBP1 early in pregnancy in mothers subsequently having a diagnosis of GDM.

There is evidence to show that early elevation in amniotic fluid glucose, which the fetus swallows, may be problematic. Early exposure to elevated glucose can lead to irreversible oxidation of albumin by 15 weeks of gestation in mothers with GDM (12) and to accelerated exhaustion of β-cells (19). Our study showed that amniotic fluid glucose was already elevated in our population with GDM by 15.2 ± 0.1 weeks of gestation, which supports a previous study (4). However, it is not yet clearly established that fetal hyperinsulinemia is actually driven by fetal hyperglycemia (4). One study showed that amniotic fluid glucose was not associated with fetal hyperinsulinism before 23 weeks of gestation (20), but another study reported increased amniotic fluid insulin in a diabetic pregnancy (6). By relating amniotic fluid glucose to insulin in contour maps, we were able to show that amniotic fluid glucose and insulin are interrelated and that elevations in both are associated with a later diagnosis of GDM.

It had been suggested that amniotic fluid insulin might be a better predictor than amniotic fluid glucose of impaired glucose tolerance in mothers with GDM (4). Insulin release by the human fetus occurs as early as 11 weeks and is found in higher concentrations in the second trimester of mothers with diabetes (21) and in those with GDM (4,6,18). Our study revealed that amniotic fluid insulin was elevated in our mothers with GDM, but amniotic fluid insulin, in contrast to these earlier studies (4,6,18), did not enter as a significant independent predictor in our multiple linear regression models when we controlled for maternal age, prepregnancy BMI, gestational age at the time of amniocentesis, and ethnicity. Each is known to modulate amniotic fluid insulin concentrations. Interestingly, when we did plot our amniotic fluid insulin with our amniotic fluid glucose in our contour maps, we also observed that our sensitivity was reasonably low (27%), but that our specificity was reasonably high (68%), which supports previous reports (4) that neither was robust enough to screen for the diagnosis of GDM. What our probability plots suggest is that using combinations of second trimester amniotic fluid glucose and insulin would more reliably "rule out" GDM than diagnose it.

IGFBP1, found in abundance in sec-

ond trimester amniotic fluid, has been associated in cord blood with an inverse relationship with birth weight in mothers with GDM at term (9). Our contour maps also showed that low amniotic fluid IGFBP1 in the presence of high glucose was associated with a higher risk of developing GDM; 30% of our mothers with GDM met these criteria. Our findings extend to amniotic fluid the previously reported relationship for low maternal plasma IGFBP1 with elevated risk of GDM (8). Prior studies have also shown that Asians are more susceptible to GDM (22). Asians reportedly have lower concentrations of plasma IGFBP1 (8). In our study population, we also observed that a higher proportion of Asians had GDM and amniotic fluid IGFBP1 was lower in these mothers with GDM. Upon controlling for ethnicity, this difference was lost, which further supports observations that Asian mothers have lower levels of amniotic fluid IGFBP1. When highly phosphorylated IGFBP1 is lower in GDM pregnancies, more free IGF-I may be available, and together these changes could account for the inverse association of IGFBP1 with birth weight (23). Whether the fundamental change represents a genetic difference or the early emergence of a metabolic dysregulation in a subset of the mothers with GDM requires further exploration.

Some limitations to the application of our findings are in order. We caution against using the exact glucose, insulin, and IGFBP1 concentrations presented in the contour maps because they were created using biobanked samples. Others have shown that there is a slight progressive decline (4%) in the concentrations of amniotic fluid glucose and an increase in insulin as a result of storage at −20°C (4). Once GDM is diagnosed, mothers receive treatment. Undoubtedly, these interventions are directed to lowering birth weight and decreasing the incidence of macrosomia. Thus, both the incidence of macrosomia and an average increase in birth weight related to GDM in our study could be underestimated. Finally, our data show that although our accuracy is reasonably high at 64–68% for each of our probability plots, our sensitivity is low (27–38%); this would indicate that there are probably other factors that are required to diagnose GDM this early in pregnancy. For now, our concentrations could be used to rule out GDM with a specificity of 68–74%.

In summary, we show that in the absence of an impaired glucose tolerance test, amniotic fluid glucose, insulin, and IGFBP1 concentrations can be perturbed by 15 weeks of gestation, some 10 weeks earlier than current screening in pregnant mothers who later develop GDM. These perturbations are also associated with an increased birth weight of at least 176 g, which may not necessarily be related to the delivery of a large-for-gestational-age infant, and, therefore, focusing on ultrasound measurements of abdominal circumference (24) although important, may not be the only clinical indicator. The possibility that combined early elevations in amniotic fluid glucose and insulin and lower IGFBP1 might be clinically useful is supported by our findings. Our results show that metabolic perturbations found in amniotic fluid early in pregnancy may underscore the higher incidence of diabetes seen in offspring of mothers with GDM later in life (11). We suggest that earlier screening for GDM using measures of amniotic fluid glucose and insulin in mothers undergoing age-related amniocentesis may be warranted.

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D.K.T. performed biochemical measures and statistical analyses, wrote the manuscript, and revised/edited the manuscript. D.H.B. interpreted data and revised/edited the manuscript. G.W.L. recruited subjects, performed amniocentesis, and revised/edited the manuscript. K.G.K. wrote the manuscript and revised/edited the manuscript.

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