# First-Trimester Follistatin-Like-3 Levels in Pregnancies Complicated by Subsequent Gestational Diabetes Mellitus

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**OBJECTIVE** — To determine whether maternal levels of follistatin-like-3 (FSTL3), an inhibitor of activin and myostatin involved in glucose homeostasis, are altered in the first trimester of pregnancies complicated by subsequent gestational diabetes mellitus (GDM).

**RESEARCH DESIGN AND METHODS** — This was a nested case-control study of subjects enrolled in a prospective cohort of pregnant women with and without GDM (≥2 abnormal values on a 100-g glucose tolerance test at ~28 weeks of gestation). We measured FSTL3 levels in serum collected during the first trimester of pregnancy. Logistic regression analyses were used to determine the risk of GDM.

**RESULTS** — Women who developed GDM ($n = 37$) had lower first-trimester serum levels of FSTL3 compared with women who did not ($n = 127$) (median 10,789 [interquartile range 7,013–18,939] vs. 30,670 [18,370–55,484] pg/ml, $P < 0.001$). When subjects were divided into tertiles based on FSTL3 levels, women with the lowest levels demonstrated a marked increase in risk for developing GDM in univariate (odds ratio 11.2 [95% CI 3.6–35.3]) and multivariate (14.0 [4.1–47.9]) analyses. There was a significant negative correlation between first-trimester FSTL3 levels and ~28-week nonfasting glucose levels ($r = -0.30$, $P < 0.001$).

**CONCLUSIONS** — First-trimester FSTL3 levels are associated with glucose intolerance and GDM later in pregnancy.

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Gestational diabetes mellitus (GDM) afflicts 4% of pregnancies in the U.S. and is associated with unfavorable perinatal outcomes (1). Although GDM is characterized by glucose intolerance, β-cell dysfunction, and insulin resistance (1–3), the pathogenesis of GDM is not well understood. The association between GDM and insulin resistance postpartum and subsequent type 2 diabetes in up to 70% of mothers (1–4) has led to the theory that GDM is simply the unmasking of a chronic condition. However, GDM resolves, at least temporarily, with the delivery of the infant and placenta, occurs more often in twin pregnancy (5), and recurs only in 30–50% of subsequent pregnancies (6), suggesting that circulating factors released by the placenta may be involved, at least partially, in its pathogenesis.

Pregnant women are typically screened for GDM at 24–28 weeks of gestation. Despite the fact that insulin sensitivity increases in the first trimester of pregnancy, a recent study showed that higher first-trimester levels of fasting blood glucose were linearly associated with increased risk of GDM, cesarean section, and macrosomia (7), suggesting that the pathophysiologic process that leads to GDM is underway weeks to months before its diagnosis. Thus, it is possible that factors linked with the pathogenesis of this condition may be present in blood samples well before the clinical diagnosis of GDM. Treatment of GDM in late pregnancy improves some adverse perinatal outcomes (8,9), but earlier detection of GDM through biomarker measurement in the first trimester of pregnancy may permit more time for intervention and lead to greater positive effects of treatment on maternal and fetal outcomes.

Follistatin-like-3 (FSTL3, also referred to as FLRG), a follistatin homolog that inhibits circulating members of the transforming growth factor-β subfamily of proteins (10), is highly expressed by the placenta (11). FSTL3 expression is increased in placentas from pregnancies complicated by intrauterine growth restriction (12). Outside of pregnancy, FSTL3 may play a major role in glucose homeostasis, as FSTL3-null mice are characterized by pancreatic β-cell hyperplasia, elevated insulin levels, increased glucose tolerance, and upregulation of hepatic gluconeogenesis (13). Further evidence supporting a role for FSTL3 in glucose homeostasis includes the biological activities of activin A and myostatin, which are antagonized by FSTL3. Activin A promotes proliferation of β-cells and secretion of insulin (14,15); activin A levels were found to be elevated in pregnancies affected by GDM in previous studies (16,17). In mouse models, the absence of myostatin promotes insulin sensitivity and protects against weight gain (18,19). Although circulating levels of myostatin during pregnancy have not been described, myostatin is expressed by the human placenta and was shown to increase glucose uptake by placental explants (20).

Based on the connection of FSTL3 to glucose homeostasis and the presence of insulin resistance and β-cell dysfunction in GDM, as well as the high expression levels of FSTL3 in the placentas of infants with small-for-gestational-age fetuses

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(12) and the occurrence of large-for-gestational-age infants in GDM, we hypothesized that circulating levels of FSTL3 would be altered in pregnancies complicated by GDM.

## RESEARCH DESIGN AND METHODS

— We conducted a nested-case control study to determine whether first-trimester FSTL3 levels were different in women who did and did not develop subsequent GDM. The parent study, the MGH Obstetrical Maternal Study (MOMS), a prospective cohort study of pregnant mothers, was described previously (21). MOMS subjects were recruited from women receiving prenatal care at Massachusetts General Hospital and affiliated health centers between 1998 and 2005. Of eligible women, 70% enrolled in the cohort at their first prenatal visit. Blood samples were collected at this time and stored at −80°C for future analysis. Clinical information including subject characteristics, glucose tolerance test results, and birth outcomes was collected from the obstetric electronic medical record. All subjects gave written informed consent, and the study protocol was approved by the Partners Human Research Committee (institutional review board).

We excluded subjects with a history of GDM in a previous pregnancy, those with preeclampsia (systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg and proteinuria), those with small-for-gestational-age infants (<10th percentile in birth weight for gestational age), those with multiple gestations, and those found to have glucose intolerance at <20 weeks of gestation, as this may represent pregestational diabetes. The standard of care at Massachusetts General Hospital is to screen all pregnant women at 24–28 weeks of gestation with a 50-g, 1-h glucose challenge test (GCT). Women with blood glucose ≥140 mg/dl 1 h after administration of the glucose challenge undergo subsequent glucose tolerance testing consisting of the administration of 100 g glucose after a 12-h fast and the measurement of blood glucose levels 0, 1, 2, and 3 h after glucose administration. GDM cases were defined as those women who had ≥2 abnormal values on glucose tolerance test (GTT) in accordance with American Diabetes Association guidelines (1). Control subjects were chosen randomly from women who passed the GCT and/or GTT. We proceeded with a 1:3 case subject–to–control subject ratio based on previous studies of biomarkers in this population (21). We focused on subjects for whom enough serum was available in the sample bank to perform FSTL3 measurements in duplicate.

Testing of serum samples was performed using a human FSTL3 DuoSet ELISA (DY1288; R&D Systems, Minneapolis, MN). Assays were run in duplicate, and the operator was blinded to the clinical data. A standard curve was generated using serial dilutions of recombinant FSTL3 (1288-F3; R&D Systems) at concentrations ranging from 10 ng/ml to 100 pg/ml in Reagent Diluent (1% BSA/PBS, DY995; R&D Systems). All reactions were performed at room temperature. Plates were coated overnight with primary mouse antihuman FSTL3 antibody in 4 μg/ml PBS (100 μl/well). On the day of the assay, each well was washed three times with 400 μl/well wash buffer (0.05% Tween 20/PBS, WA126; R&D Systems), using a Columbus Pro washer (Tecan) and then blocked with 300 μl/well Reagent Diluent for 1 h. Plates were then washed three times with wash buffer, and 100 μl standard or sample was added to each well. Plates were incubated for 2 h on a table-top shaker and washed three times as described above. Then 400 ng/ml secondary biotinylated mouse antihuman FSTL3 antibody (100 μl/well) was added, and plates were incubated for 2 h on a table-top shaker. Plates were washed again with wash buffer three times, and streptavidin conjugated to horseradish peroxidase (100 μl/well) was added for 20 min. Plates were washed three times in wash buffer, and color reagent containing $H_2O_2$ and tetramethylbenzidine (100 μl/well) was added (DY993; R&D Systems). Plates were incubated for 20 min and stop solution (2 N $H_2SO_4$, 50 μl, DY994; R&D Systems) was added to each well. Optical density was measured using a microplate reader (model 660; Bio-Rad Laboratories). Optical density at 550 nm was subtracted from optical density at 450 nm, and serum concentrations were determined using a standard curve generated by a quadratic plot of the standard sample concentrations versus the measured optical density. The interassay coefficient of variation for this assay was 10.5%.

Subject characteristics and levels of FSTL3 in case and control subjects were compared using *t* tests, Wilcoxon tests, or $\chi^2$ tests as appropriate. Within the control group, one-way ANOVA was performed to determine whether FSTL3 levels differed in women who had failed the GCT but passed the GTT. Subjects were divided into tertiles based on FSTL3 levels, and odds ratios for the development of GDM were calculated for each tertile. Logistic regression analysis with appropriate indicator variables was performed to create univariate and multivariate models for the odds of developing GDM. Spearman correlations between FSTL3 levels, blood glucose levels after a 50-g glucose challenge, and other subject characteristics were sought. Statistical analyses were performed with the use of SAS 9.0 (SAS Institute, Cary, NC).

**Table 1—*Characteristics of subjects with and without GDM at prenatal visit and delivery***

| | GDM | Control | *P** |
|---|---|---|---|
| *n* | 37 | 127 | |
| First prenatal visit | | | |
| Gestational age (weeks) | 10.5 ± 0.32 | 11.0 ± 0.15 | 0.20 |
| Age (years) | 34.2 ± 0.86 | 34.0 ± 0.41 | 0.80 |
| BMI (kg/m$^2$) | 29.2 ± 1.4 | 26.8 ± 0.49 | 0.20 |
| Systolic blood pressure (mmHg) | 114 ± 1.6 | 112 ± 1.05 | 0.32 |
| Diastolic blood pressure (mmHg) | 72 ± 1.4 | 70 ± 0.73 | 0.09 |
| % nulliparous | 46 | 42 | 0.65 |
| % Caucasian | 65 | 81 | 0.04† |
| Delivery | | | |
| Gestational age (weeks) | 39.0 ± 0.20 | 39.8 ± 0.10 | <0.001† |
| Weight gain (lb) | 22.3 ± 1.9 | 27.5 ± 1.3 | 0.005† |
| Birth weight (g) | 3,491 ± 91 | 3,538 ± 43 | 0.62 |
| Cesarean section (%) | 30 | 28 | 0.80 |

Data are means ± SEM or %. *P value reflects *t* test for normally distributed variables (age, gestational age, and birth weight), Mann-Whitney-Wilcoxon test for nonnormally distributed variables (BMI, blood pressure, and weight gain), and $\chi^2$ test for categorical variables (race, parity, and cesarean section). †Significant at $P < 0.05$ level.

**RESULTS** — Characteristics of the 37 subjects who developed GDM and the

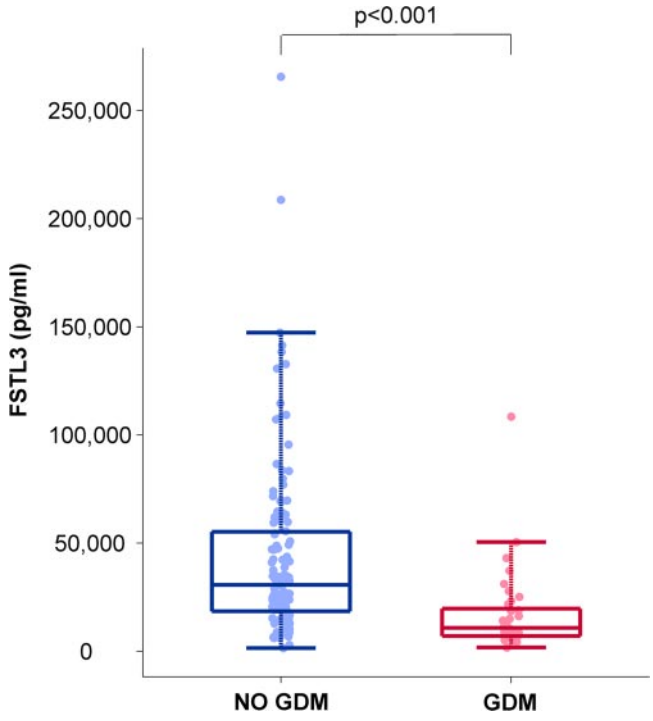


**Figure 1**—*First-trimester FSTL3 levels in women who did and did not develop GDM. FSTL3 levels were measured in serum collected from women with GDM and control subjects at the first prenatal obstetric visit. FSTL3 levels were significantly lower in women who developed GDM (*P *< 0.001). Box plots depict the median (horizontal line in each box), the 25th percentile (bottom of each box), and the 75th percentile (top of each box). Box whiskers extend to the highest/lowest nonoutlier value. Outliers were defined as lying greater than three interquartile ranges outside the 25th or 75th percentile. Scatter plot overlay depicts levels of FSTL3 in individual subjects.*

127 control subjects are presented in Table 1. Subjects were similar in age and parity and had similar blood pressures measured at the first prenatal visit. Gestational age at the time of blood collection was similar in both groups. Subjects who developed GDM had slightly greater BMI compared with those who did not, but this difference was not statistically significant. There was a greater percentage of Caucasians in the control group than in the GDM group. Women with GDM delivered earlier in gestation than control women, and women with GDM gained less weight between the first visit and delivery. Infant birth weight and percentage of women undergoing cesarean section did not differ between the groups.

First-trimester FSTL3 levels were lower in subjects who developed GDM than in control subjects (median 10,789 [interquartile range 7,013–18,939] vs. 30,670 [18,370–55,484] pg/ml, $P < 0.001$) (Fig. 1). Average FSTL3 levels in subjects who failed the GCT but passed the GTT was 27,313 [20,609–54,169] pg/ml, lower but not significantly different from FSTL3 levels in women who passed the GCT (31,066 [18,039–59,407] pg/ml, $P = 0.614$) and significantly greater than those who went on to develop GDM ($P < 0.001$). When subjects were divided into tertiles based on FSTL3 levels, women in the first tertile had an 11.2-fold risk of developing GDM compared with women in the third tertile (Fig. 2). This odds ratio increased to 14.0 after adjustment for gestational age at blood collection, maternal age, race, BMI, blood pressure, and parity. In the multivariate logistic regression model, only FSTL3 tertile and nonwhite race were significant independent predictors of GDM.

FSTL3 was not correlated with gestational age at blood collection ($P = 0.44$), BMI ($P = 0.29$), systolic blood pressure ($P = 0.87$), diastolic blood pressure ($P = 0.96$), nonwhite race ($P = 0.40$), or maternal age ($P = 0.71$). There was no difference between levels of FSTL3 in white and nonwhite women ($P = 0.90$), between nulliparas and multiparas (0.70), or between women with different smoking histories ($P = 0.80$). Blood glucose 1 h after a 50-g glucose load (GCT result) was inversely correlated with FSTL3 level ($r = -0.30$, $P < 0.001$) (Fig. 3) and positively correlated with BMI ($r = 0.18$, $P = 0.03$) and nonwhite race ($r = 0.23$, $P = 0.003$). In a multivariate linear regression model, only FSTL3 and nonwhite race independently predicted GCT result.

**CONCLUSIONS** — In this nested case-control study, we showed that low FSTL3 levels in the first trimester are associated with the development of glucose intolerance and GDM later in pregnancy. When subjects were divided into tertiles based on FSTL3 levels, women with the lowest levels demonstrated a markedly elevated risk compared with women with the highest levels. This estimate of risk was further increased after adjustment for age, BMI, nonwhite race, blood pressure, and multiparity, which are known risk factors for GDM (22). Levels of FSTL3 were inversely correlated with levels of blood glucose during a 50-g GCT. These data are consistent with, but do not prove, involvement of FSTL3 in GDM pathophysiology. The association between first-trimester FSTL3 levels and subsequent GDM may allow FSTL3 to be tested as a candidate biomarker for estimation of GDM risk early in pregnancy.

We expect that our sample is representative of the pregnant population treated at a large New England hospital, with the exception that no subject had a previous history of GDM. Of the subjects, 23% were ethnic/racial minorities. Subjects with and without GDM were similar with the exception of race, gestational age at delivery, and weight gain during pregnancy. Nonwhite race is a known risk factor for GDM (22); this may explain the difference in racial makeup of the case and control groups. Women with GDM are followed closely for fetal growth and delivery is targeted if there is concern for macrosomia (1). This probably accounts for the younger gestational age at delivery, lower weight gain, and similar birth weights in the GDM group. FSTL3 levels were not correlated with BMI, parity, maternal age, nonwhite race, or smoking history, clinical factors known to be associated with GDM (22). In our sample, BMI, parity, maternal age, and smoking history were not significantly associated with a higher risk of GDM, implying that our study may have been underpowered to detect these known relationships and any relationship between these factors and FSTL3. In contrast, FSTL3 level and nonwhite race predicted GDM independently, implying that FSTL3 levels in

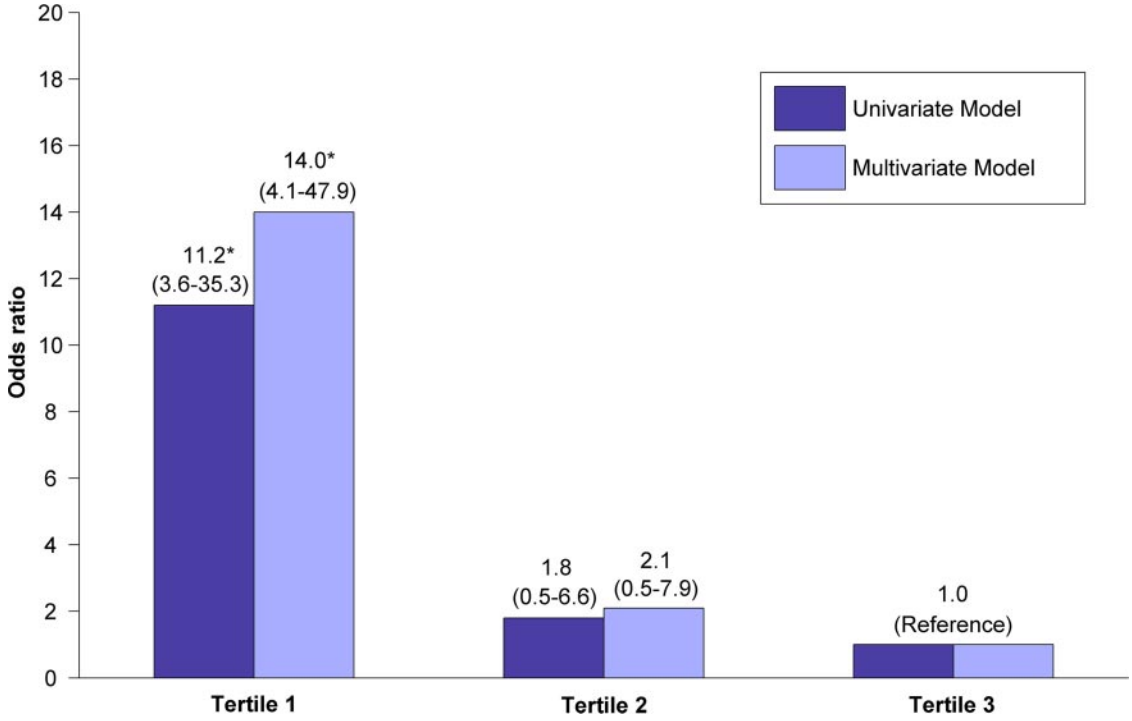


**Figure 2**—*Odds of developing GDM by first-trimester FSTL3 tertile. Subjects were divided into tertiles based on the first-trimester FSTL3 level. Univariate (dark blue) and multivariate (light blue) logistic regression analyses were used to determine odds ratios for the development of GDM in each tertile. The multivariate logistic regression model includes adjustment for age, gestational age, diastolic blood pressure, BMI, nonwhite race, and multiparity. *Significantly different from the reference tertile at the* $P < 0.05$ *level.*

nonwhite women would not completely explain the reason for their known elevated risk for GDM.

Because the placenta expresses high levels of FSTL3, we suspect it to be the major source of FSTL3 in the maternal serum, but we did not test this hypothesis. FSTL3 is also expressed at a moderate level by the pancreas and other adult tissues. Given the pancreatic hypertrophy and hypersecretion that occur during pregnancy, we cannot rule out the pancreas or other maternal tissues as a source of circulating FSTL3.

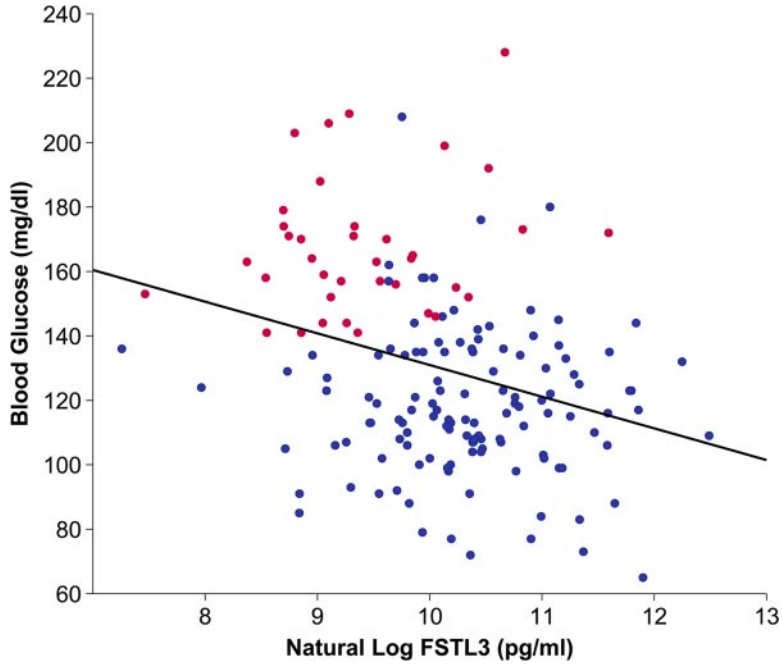


**Figure 3**—*Relationship between first trimester FSTL3 and GCT result. Glucose challenge test (GDM screening) was performed at 24–28 weeks of gestation. A 50-g glucose load was administered orally, and blood glucose was measured after 1 h.* $r = -0.30$, $P < 0.001$ *(Spearman correlation). Red markers represent GDM case subjects; blue markers represent control subjects.*

Although we did not test the validity of potential pathophysiological mechanisms operating in GDM, our finding of low FSTL3 levels in pregnancies complicated by GDM suggests that FSTL3 may be involved in glucose metabolism during pregnancy. The third trimester of normal pregnancy is characterized by insulin resistance, which is matched by pancreatic β-cell hyperplasia and increased insulin secretion (3), resulting in normal glucose tolerance. The signals leading to upregulation of pancreatic β-cell performance during pregnancy are unknown. However, the action of activin A on pancreatic β-cells (14,15), our finding of low FSTL3 levels in the serum of women with GDM, and the previous reports of elevated activin A levels in pregnancies affected by GDM (16,17) underscore a possible role for activin A in the promotion of islet cell hyperplasia and increased insulin secretion during pregnancy.

The insulin resistance of late pregnancy may convey an adaptive advantage to the fetus in terms of energy acquisition and is thus presumed to be caused by a combination of placental hormones including human placental lactogen; however, most studies have not found a correlation between levels of previously studied placental hormones and insulin resistance in pregnant women (23). Because GDM is characterized by insulin resistance in excess of normal pregnancy (1–3), GDM may represent a maternal-fetal conflict over energy resources, and yet unexplored factors released by the placenta may be involved in its pathogenesis. Myostatin, whose absence is known to promote insulin sensitivity and protect against weight gain (18), is released by the placenta during pregnancy in unknown amounts (20). Thus, myostatin may be a candidate protein for contribution to the insulin resistance of pregnancy and/or GDM. We speculate that low levels of FSTL3 may be involved in GDM physiology by either leading to disinhibition of myostatin, promoting excessive insulin resistance, or leading to disinhibition of activin A, promoting compensatory insulin secretion in the setting of hyperglycemia.

FSTL3 levels were inversely correlated with glucose challenge screening test results, underscoring the possible clinical utility of FSTL3 measurement early in pregnancy. Of note, our study does not indicate how FSTL3 levels compare in utility to other screening tests for GDM. With the GTT as a gold standard, one large study showed that the GCT had a sensitivity of just 73% (24). It is possible that women with low FSTL3 levels who passed the GCT might have had an abnormal GTT if such testing had been performed. Consistent with this possibility, our data show that women who failed the GCT but passed the GTT had FSTL3 levels similar to those of women who passed the GCT. Measurement of FSTL3 in a sample in which all subjects undergo glucose tolerance testing would provide a better estimation of the sensitivity and specificity of FSTL3 testing. In our sample, FSTL3 testing with a cutoff point of 50,000 pg/ml could have eliminated the GCT for 29% of women, while still recognizing 95% of women with GDM identified by routine screening. Given current U.S. practices of universal screening and the controversy surrounding selective screening based on risk factors (25), this finding has potential cost-saving implications.

Strengths of our study include prospective data collection and the large amount of clinical data available to us through the MOMS study. Limitations include the lack of longitudinal data on FSTL3 levels and the lack of glucose tolerance testing in every participant. Measurement of FSTL3 levels throughout pregnancy would provide a context in which to interpret FSTL3 levels. A longitudinal study of FSTL3 levels could also provide information as to whether FSTL3 is a marker of insulin sensitivity during pregnancy and whether FSTL3 levels increase with increasing placental mass. Although our data provide evidence of an association between low first-trimester FSTL3 levels and GDM, the involvement of FSTL3 in any causal or compensatory pathway has not been proven. Investigation of the role of FSTL3 in glucose homeostasis may provide insights into GDM pathogenesis and should be undertaken using animal models. Ultimately, measurement of FSTL3 level may be a means to distinguish women at high and low risk for GDM early in pregnancy, but clinical trials using improved automated assays will be necessary before implementation of any future clinical laboratory test based on the measurement of FSTL3.

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No potential conflicts of interest relevant to this article were reported.

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