Deregulated Levels of Vascular Endothelial Growth Factor, Tumor Necrosis Factor-α and Total Cholesterol Early in Pregnancy may Predict Oncoming Gestational Diabetes Mellitus

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Abstract

Objectives: Evaluation of the relationship between maternal glycemia and lipidemic statuses, serum levels of vascular endothelial growth factor (VEGF) and tumor necrosis factor-α (TNF-α) at the 6th gestational week (GW) and the development of gestational diabetes mellitus (GDM) among non-diabetic pregnant women.

Patients & Methods: 169 newly pregnant women, 20 non-pregnant/non/diabetic (NP/ND) and 20 non-pregnant/diabetic (NP/D) women underwent 75-Oral glucose tolerance test (OGTT) and gave blood samples for estimation of blood levels of glycosylated hemoglobin A1c (HbA1c), plasma lipid profile and serum levels of VEGF and TNF-α at the 6th GW of the pregnant women and the 24th GW, OGTT was repeated to define GDM women.

Results: At the 24th GW, OGTT defined 37 women had GDM and 132 were non-GDM. At the 6th GW, NP/D and GDM women had significantly higher fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), and very low-density lipoprotein (VLDL) with significantly lower high-density lipoprotein (HDL-c) compared to NP/ND women, but differences were non-significant between non-GDM and NP/ND women. At-enrolment means serum levels of TNF-α were significantly lower in samples of NP/ND than in samples of women of other groups that showed non-significant differences. On contrary, at-enrolment mean serum levels of VEGF were significantly higher in NP/D and GDM women, while were non-significantly higher in non-GDM women compared to NP/ND. Moreover, at-enrolment serum VEGF levels were significantly lower in pregnant women compared to NP/D women with significantly lower levels in non-GDM than GDM women. Statistical analyses defined high at-enrolment plasma TC and serum VEGF and TNF-α as the most significant predictors for GDM during pregnancy progress. Kaplan-Meier Regression analysis showed that the risk for the development of GDM was increased by 50% at a plasma TC level of 217 mg/ml (95%CI: 215.2-218.8), serum VEGF at 137 pg/ml (95%CI: 132.2-151.8) and TNF-α level at 3.49 (95%CI: 3.35-3.97).

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Conclusion: GDM may be related to the interplay between high levels of VEGF, inflammatory cytokines, and hyperlipidemia. High blood levels of VEGF and TC can discriminate against women vulnerable to developing GDM with high sensitivity, specificity, and negative predictive values.

Keywords: Gestational diabetes mellitus, Vascular endothelial growth factor, Tumor necrosis factor-α, Lipid profile.

Introduction

Gestational diabetes mellitus (GDM) is pregnancy-induced diabetes despite being a temporary form; it is potentially associated with maternal, fetal, and neonatal complications [1]. Unfortunately, the incidence of GDM is increasing globally [2], and failure of diet and exercise as management for GDM necessities a shift to pharmacotherapy [3].

The progressively increasing incidence of GDM parallels the increasing incidence of obesity which was considered an epidemic [4]. Moreover, obesity and abdominal adiposity are two of the four components of metabolic syndrome, which is a worldwide problem, and the other two are hyperglycemia and hypertension; these components of metabolic syndrome illustrate the relation between obesity and diabetes [5].

Vascular endothelial growth factor (VEGF) is a 45 kDA glycoprotein, homodimeric, basic, and able to bind heparin. The VEGF family comprised several members; VEGF A to E [6]. VEGF is a signal protein produced by many cells to stimulate angiogenesis, especially VEGF-A, which consists of 121 amino acids and plays an important role in neo-angiogenesis and its increased levels indicate the intensity of neoangiogenesis [7].

Tumor necrosis factor alpha (TNF-α), is a pro-inflammatory cytokine that is produced by macrophages/monocytes during acute inflammation and plays a diverse range of signaling events within cells [8]. TNF-α acting as a trimer exerts many of its effects by binding to cell membrane receptors to regulate a diverse range of physiological processes [9].

Objectives

The study tried to evaluate the relationship between maternal glycemic, angiogenic, and inflammatory statuses and the development of GDM among non-diabetic pregnant women.

Design:

Prospective comparative study

Setting

Obstetrics & Gynecology Department, Faculty of Medicine, Benha University

Ethical consideration

The was started in Nov 2021 after the preliminary approval of the study protocol by the Local Ethical Committee and the final approval was obtained after the finalization of case collection and follow-up period by RC: 5.9.2022.

Participants

All newly pregnant women who attended within the 1st six gestational weeks (GW) the outpatient clinic of Obstetrics & Gynecology at Benha University Hospital were evaluated for the presence of exclusion and inclusion criteria. Evaluation encompassed the collection of demographic data, full medical and obstetric history taking, and clinical examination. Then, all women underwent abdominal ultrasonography for assurance of the presence of a singleton fetal sac containing a viable fetus and gave blood samples for determination of their glycemic status and other investigations. All enrolled women were asked to attend the outpatient lab fasting for more than 12 hours to give a blood sample for estimation of their plasma lipid levels. The study also included 40 non-pregnant (NP) women of cross-matched age and BMI; 20 women were diabetics (NP/D) as a positive control group and 20 non-diabetic women (NP/ND) as a negative control group. Control women must be free of inclusion and exclusion criteria, and accepted to undergo a full profile of investigations.

Exclusion criteria

It included the presence of body mass index (BMI) ≥ 30 kg/m^2, metabolic syndrome, genetic hypercholesterolemia, and hepatic, cardiac, and vascular diseases.

Inclusion criteria

Inclusion criteria are attendance early in pregnancy within the 1st 6 GW, absence of exclusion criteria, acceptance for the study participation, and attendance at the start of the 24th GW to give blood samples for re-evaluation of glycemic status to define women who developed GDM.

Evaluation Tools:

1. **The 75-oral glucose tolerance test (OGTT):** The enrolled patients underwent the OGTT at the time of enrolment and the start of the 24th GW. Fasting blood glucose (FBG) and 2-h postprandial blood glucose (PPBG) were estimated. PPBG has to be estimated after receiving 75 g glucose by 2-h. GDM was diagnosed if the FBG and 2-h PPBG levels were ≥ 92 and ≥ 153 mg/dl, respectively [10].

2. **Estimated levels of HbA1c:** HbA1c at the cutoff point of 6% differentiates between diabetic (>6%) and non-diabetic ($\leq 6\%$) states [11].

Investigations

- The 1st patients and controls' blood samples that were obtained at the time of enrolment were divided into three parts:

1. The 1st part was collected in a fluoride-containing tube (2:1=NaF: blood, by vol.) to preserve blood glucose (BG) until being sent for estimation of BG at the hospital lab by glucose oxidase method [12].

2. The 2nd part was collected in EDTA containing tube for HbA1c estimation using Latex Turbidimetry (LINEAR CHEMICALS S.L. Joaquim Costa, Montgat, Barcelona, Spain) [13].

3. The 3rd part was collected in a plain tube, allowed to clot, and centrifuged to separate serum that was collected in an Eppendorf tube and frozen to -20oC till being ELISA assayed for estimation of serum levels of tumor necrosis factor-α (TNF-α) and

vascular endothelial growth factor (VEGF) using ELISA kit for estimation of human levels of these biomarkers (Abcam Inc., San Francisco, USA; catalog ab181421, ab100662, respectively) according to manufacturer instructions [14, 15].

- The 2nd patients and controls' blood samples that were obtained 12-h after enrolment were collected in EDTA containing tube for estimation of total cholesterol, low, very-low, and high-density lipoprotein cholesterol (LDL-c, VLDL-c, and HDL-c), and triglycerides (TG) using :

Study outcome

The possible relationship between the estimated parameters at enrolment time and the development of GDM at the 24th GW was evaluated.

Statistical analysis

The results were analyzed using One-way ANOVA, paired t-test, and Chi-square test (X^2 test). Regression analysis and the Receiver characteristic curve were used to determine the significant predictors as judged by the area under the curve (AUC). The suggested cutoff points using the median and quartile values were evaluated using the Kaplan-Meier regression analysis using IBM® SPSS® Statistics (Version 22, 2015; Armonk, USA) for Windows statistical package. P value <0.05 was considered statistically significant.

Results

During the study period, 218 women had attended the outpatient clinic within the 1st 6 GW, but 21 had BMI ≥ 35 kg/m2, 7 women were maintained on cholesterol adjusting therapies, 4 were hypertensive, 3 patients had a history of hepatitis, and 2 had renal manifestations, and 181 women were enrolled in the study, 12 women did not attend at the 24th GW and were excluded from the study and the data of 169 women were analyzed. At the 24th GW, lab re-evaluation detected 37 women who developed GDM (GDM group), while in 132 women 24-GW levels of BG did not reach the diagnostic level for GDM (Non-GDM group) as shown in figure 1. Enrolment data showed a non-significant difference between the women of the study groups (Table 1).

Table (1): Patients' enrolment data

Group Data	NP/ND (n=20)	NP/D (n=20)	GDM (n=132)	Non-GDM (n=37)
Age (years)	29.5±2.9	29.2±3.2	29±3.4	29±3.5
Weight (kg)	83±5.5	84.1±6.4	81.3±3.7	84.5±6
Height (cm)	168.8±4.8	169.1±5.6	169.1±3.1	168.9±2.9
BMI (kg/m^2)	29.1±1.1	29.4±2.1	28.5±1.8	29.6±1.8‡
Gravidity	1.75±0.8	1.8±0.8	1.7±0.6	1.7±0.6
Parity	1.3±0.5	1.3±0.7	0.7±0.6	0.7±0.6
Systolic BP (mmHg)	114.8±8.6	115.2±9.4	115.2±5.6	116±5.8
Diastolic BP (mmHg)	77.7±5	77.8±5.9	78±4.7	77.6±4.4

Mean and standard deviation; BMI: Body mass index; BP: Blood pressure
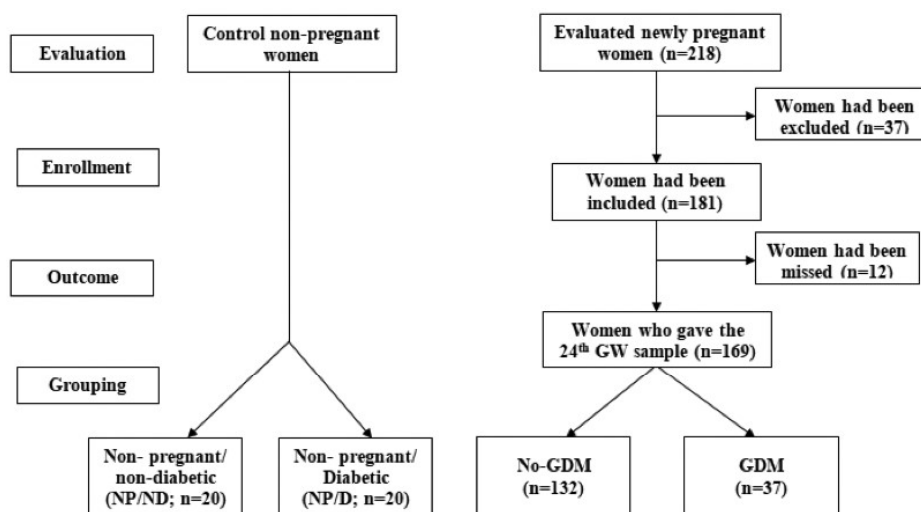
Fig. (1): Study Flow Chart

At the time of enrolment, women of NP/D and GDM had significantly (P<0.001) higher TC, TG, and VLDL with significantly lower HDL-c lipids in comparison to NP/ND women, while estimated levels of these lipids showed non-significant differences between non-GDM and NP/ND women. Plasma TC, TG, and VLDL levels were significantly higher in NP/D compared to non-GDM women, with non-significant differences concerning plasma HDL-c and LDL-c levels between women of both groups. Plasma levels of TC, TG, and VLDL estimated in women of the GDM group were significantly higher than that of the non-GDM group, with non-significant differences as regards plasma LDL-c and HDL-c. GDM women showed significantly lower plasma levels of TG compared to NP/D women (Table 2, Fig. 2).

Table (2): Lipid profile of enrolled women

Group Data	NP/ND (n=20)	NP/D (n=20)	GDM (n=132)	Non-GDM (n=37)
TC (mg/dl)	180±16.5	205.4±23.4*	179.1±20.2†	202.8±22.6*‡
TG (mg/dl)	50.2±8.8	71.3±7.4*	52±5.4†	65.2±9.2*†‡
HDL-c (mg/dl)	46±3.5	40.1±4.9*	43.1±4.7	41.5±7.3*
LDL-c (mg/dl)	65.4±8.9	68.6±19.2	64±22.2	70±25.5
VLDL (mg/dl)	18.4±6.5	25.4±7.9*	20±4.8†	26.8±4.7*‡

Mean and standard deviation; *: significant difference versus non-diabetic non-pregnant women; †: significant difference versus diabetic non-pregnant women; ‡: significant difference versus non-GDM; symbols indicate significance at P<0.001

Fig. (2): Mean plasma levels of lipids estimated in enrolled women at time of enrolment
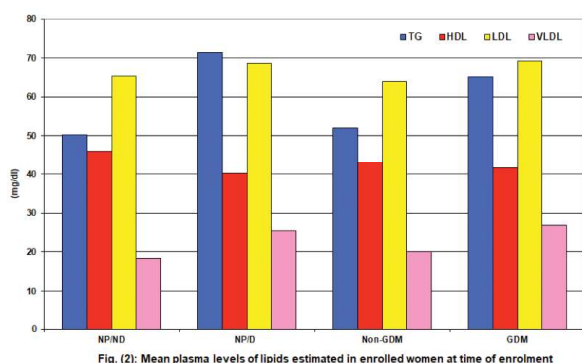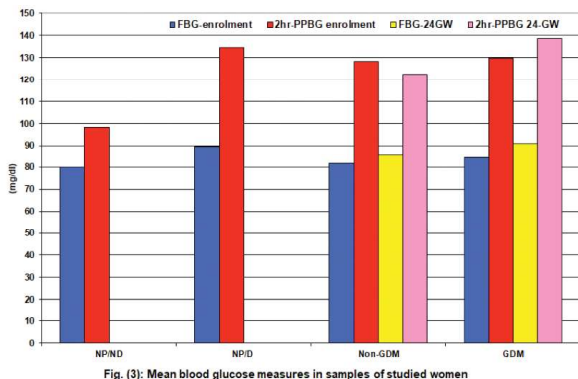
Enrolment FBG levels were significantly lower in samples of NP/ND women compared to levels estimated in samples of women of NP/D and GDM women but were non-significantly lower than levels estimated in non-GDM women. On the other hand, at-enrolment FBG levels in samples of NP/D were significantly higher than that of both groups of pregnant women with significantly higher levels in samples of GDM women. On contrary, 2-hr PPBG levels were significantly lower in NP/ND women compared to women of other groups, and in samples of pregnant women compared to in samples of NP/D with non-significant differences between pregnant women. Mean levels of FBG and 2-h PPBG estimated in 24-GW samples of non-GDM women were significantly higher than in samples of NP/ND women but were significantly lower than in samples of NP/D and GDM women. However, mean levels of FBG estimated in 24-GW samples of GDM women were significantly and non-significantly higher than in samples of NP/ND and NP/D women, respectively. On the other hand, 2-h PPBG levels in 24-GW samples were significantly higher in samples of pregnant than in samples of non-pregnant women (Fig. 3). At enrolment mean HbA1c% in NP/ND was significantly higher than that of women of other groups and that determined at 24-GW of non-GDM women but was non-significantly higher than percentage determined at 24-GW of GDM women. Mean HbA1c% in GDM at enrolment and 24-GW was significantly higher in comparison to that of non-GDM, and both were significantly higher than the HbA1c% of NP/ND women (Table 3).

Table (3): Glycemic status of studied women that was assessed at the time of enrolment and time of diagnosis of GDM for pregnant women

Group Data		NP/ND (n=20)	NP/D (n=20)	GDM (n=132)	Non-GDM (n=37)
Enrolment 75-OGTT (mg/dl)	FBG	80±4.4	89.6±5.3*	81.7±4†	84.5±3.4†‡
	2-h PPBG	98.2±5.8	134.6±8.2*	128.4±7.4*†	129.6±7*†
24-GW 75-OGTT (mg/dl)	FBG			85.6±3.5*†	90.9±6.1*‡
	2-h PPBG			122.4±9.2*†	138.7±22*†‡
HbA1c (%)	Enrolment	4.47±0.5	6.9±1.3*	4.5±0.5†	5.1±0.9*†‡
	24-GW			5.5±0.6*†	6.4±1.2*‡

Mean and standard deviation; *: significant difference versus non-diabetic non-pregnant women; †: significant difference versus diabetic non-pregnant women; ‡: significant difference versus non-GDM; symbols indicate significance at P<0.001

Fig. (3): Mean blood glucose measures in samples of studied women

At-enrolment means serum levels of TNF-α in a sample of NP/ND were significantly lower than in samples of women of other groups with non-significant differences between women and the later women. At-enrolment means serum levels of VEGF were significantly higher in NP/D and GDM women, while were non-significantly higher in non-GDM women compared to NP/ND. Moreover, at-enrolment serum VEGF levels were significantly lower in pregnant women compared to NP/D women with significantly lower levels in non-GDM than in GDM women (Table 4).

Table (4): Serum levels of TNF-α and VEGF estimated in enrolment samples of women of studied groups

| Group Data	NP/ND (n=20)	NP/D (n=20)	GDM (n=132)	Non-GDM (n=37)
TNF-α (ng/ml)	1.9±0.46	2.67±0.79*	2.7±0.81*	3.14±0.86*
VEGF (pg/ml)	107.5±14.2	160±22.2*	100.6±20.7†	129±34.2*†‡

Mean and standard deviation; *: significant difference versus non-diabetic non-pregnant women; †: significant difference versus diabetic non-pregnant women; ‡: significant difference versus non-GDM; symbols indicate significance at $P<0.001$

The ROC curve analysis for the at-enrolment data as predictors for GDM development during pregnancy stratified these data as shown in table 4 and figure 4 according to the significance of the calculated area under the curve (AUC). Linear regression analysis for the variate with significant AUC defined high plasma TC and serum VEGF and TNF-α as the most significant predictors for GDM during pregnancy progress.

Table (5): Statistical analyses of enrolment data of pregnant women as predictors for the development of GDM at the 24th GW

Methods Variate	ROC Curve analysis				Regression analysis	
	AUC	SE	P	95% CI	β	P
Age (years)	0.482	0.046	0.700	0.392-0.572	0.055	0.932
BMI (kg/m²)	0.643	0.043	0.002	0.558-0.728	0.168	0.010
plasma TC (mg/dl)	0.776	0.038	<0.001	0.701-0.852	0.338	<0.001
Serum VEGF (pg/ml)	0.731	0.042	<0.001	0.649-0.812	0.287	<0.001
Serum TNF-α (ng/ml)	0.635	0.004	0.003	0.548-0.721	0.166	0.008

ROC curve: Receiver Operating Characteristics curve; AUC: Area under the curve; CI: Confidence interval; β: Standardized coefficient; BMI: Body mass index; TC: Total cholesterol; VEGF: Vascular endothelial growth factor; TNF-α: Tumor necrosis factor-α
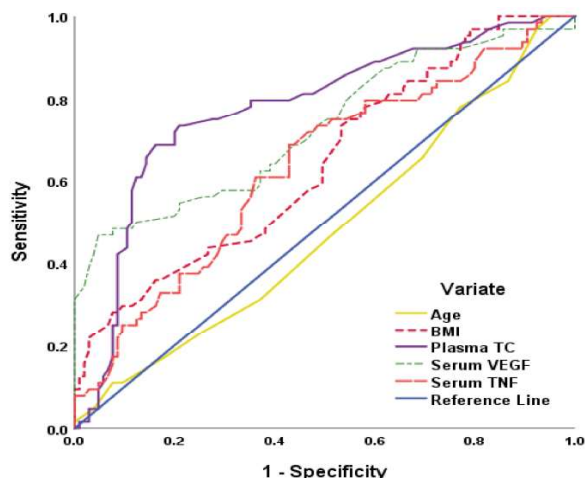
Fig. (4): ROC curve analysis for at-enrolment data for the development of GDM at the 24ᵗʰ GW

Kaplan-Meier Regression analysis showed that the risk for the development of GDM was increased by 50% at a plasma TC level of 217 mg/ml (95%CI: 215.2-218.8), serum VEGF at 137 pg/ml (95%CI: 132.2-151.8) and TNF-α level at 3.49 (95%CI: 3.35-3.97) as shown in figure 5a-c.
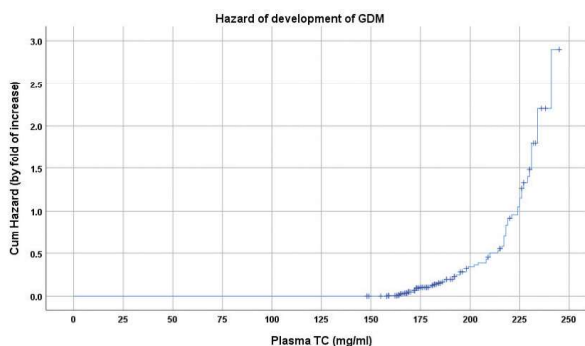
Fig. (5a): Kaplan-Meier regression hazard curve for at enrolment plasma TC for development of GDM at the 24ᵗʰ GW
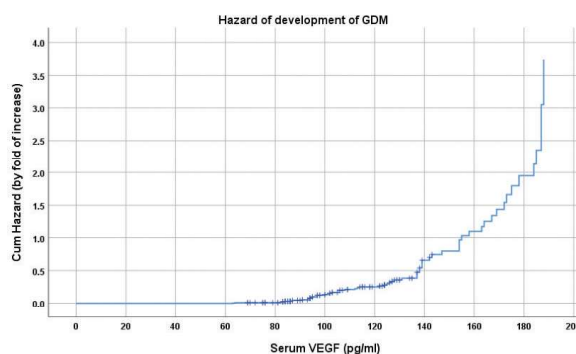
Fig. (5b): Kaplan-Meier regression hazard curve for at enrolment serum VEGF for development of GDM at the 24ᵗʰ GW
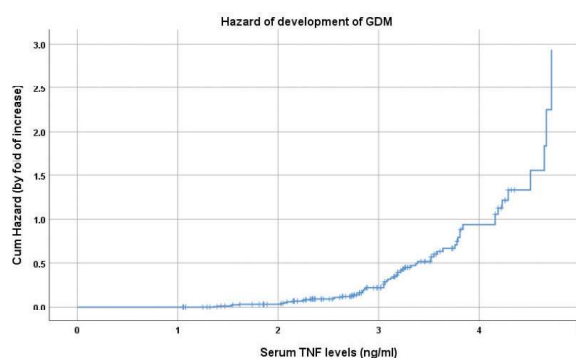
Fig. (5c): Kaplan-Meier regression hazard curve for at-enrolment serum TNF-α for development of GDM at the 24ᵗʰ GW

Test validity evaluation defined high serum VEGF at a cutoff point of 137 pg/ml had the highest sensitivity and negative predictive value for discriminating women liable to develop GDM during pregnancy, while high TC level at a cutoff point of 217 mg/ml had the highest specificity and both had nearly equal accuracy of prediction.

Table (6): Test validity characters of the cutoff points of the predictors for the development of GDM at the 24ᵗʰ GW

	Plasma TC (217 mg/ml)	Serum VEGF (137 pg/ml)	Serum TNF-α (3.49 ng/ml)
Sensitivity (%)	74.3 (95% CI: 56.7-87.5)	85.7 (95% CI: 69.7-95.2)	53.9 (95% CI: 37.2-69.9)
Specificity (%)	78.7 (95% CI: 70.4-85.6)	74.6 (95% CI: 66.4-81.7)	66.9 (95% CI: 58.1-74.9)
PPV (%)	50 (95% CI: 40.3-59.7)	46.9 (95% CI: 39-97.8)	32.8 (95% CI:25-41.7)
NPV(%)	91.4 (95% CI: 85.8-95)	95.2 (95% CI: 89.8-97.8)	82.9 (95% CI: 77.1-87.4)
Accuracy (%)	77.7 (95% CI: 70.4-84)	76.9 (95% CI: 69.8-83)	63.9 (95% CI: 56.2-71.1)

PPV: Positive Predictive Value; NPV: Negative Predictive Values; TC: Total cholesterol; VEGF: Vascular endothelial growth factor; TNF-α: Tumor necrosis factor-α

Discussion

The estimated glycemic parameters showed deterioration of glucose homeostasis with pregnancy as evidenced by the higher BG levels and HbA1c% at the 24th GW concerning the at-enrolment levels. These findings point to the diabetogenic phenotype of pregnancy, irrespective of reaching the diagnostic level of GDM or not, and go in hand with the previous animal models that approved the diabetogenic nature of pregnancy [16, 17].

This diabetogenic status of pregnancy was recently attributed by Amabebe & Anumba [18] to the alteration of gut microbiota which is responsible for the promotion of the metabolic changes required by the mother and fetus and in late pregnancy these microbiota shifts maternal metabolism in a diabetogenic direction, but if these alterations occurred earlier during pregnancy, the diabetogenic phenotype may develop during the second trimester resulting in GDM status.

At the 24th GW, 37 women developed GDM, while BG levels of the remaining 1325 women were under the diagnostic levels for GDM. This finding indicated the individual variations in response to the glycemic effect of pregnancy and was explained by Artunc-Ulkumen et al. [19] on genetic bases due to under-expression of the "a disintegrin and metalloproteinase with thrombospondin motifs-9" (ADAMTS-9) gene in GDM women than in non-GDM women. Recently, Lu et al. [20] detected placental methylation and expression profiles that mirror the molecular characteristics of IR and T2DM in the placentas of women who developed GDM. Moreover, Franzago et al. [21] found DNA methylation levels at CpG1 on the maternal side of the placentas were positively related to 2-h PPBG on 75-OGTT. On the other hand, Karagoz et al. [22] attributed the development of GDM to increased levels of apelin that inhibit the sodium-dependent glucose transporter leading to reduced intracellular glucose transport with subsequent hyperglycemia.

The detected lower serum levels of VEGF in samples of non-GDM women in comparison to NP/ND women indicated that normal pregnancy is associated with decreased levels of VEGF. On the other hand, in samples of GDM women, VEGF levels were higher than in NP/ND and non-GDM women, thus pointing to a possible relation between disturbed glucose homeostasis in the diabetogenic direction and increased levels of VEGF. In support of this assumption, serum VEGF levels were significantly higher in NP/D women than in women of other groups.

These findings go in hand with previous studies that reported higher levels of VEGF in GDM women than in control pregnant or non-pregnant women [23, 24]. Further, Sirico et al. [25] detected higher expression levels of VEGF in the placentas of GDM women than in controls, and the VEGF positivity was associated with the presence of GDM. In a trial to explain the pathogenesis of this relation; Dong [26] found a significant correlation between the risk of GDM and higher VEGF polymorphisms and its expression and Shi et al. [27] detected upregulated expression levels of placental receptor for advanced glycation end products and VEGF in GDM placentas. Recently, the reported relation was attributed by Zheng et al. [28] to the downregulation of expression levels of microRNA195-5P that was detected to be negatively correlated to VEGF levels in GDM mouse placental tissues.

Serum levels of TNF-α were significantly higher in pregnant and NP/D women than in NP/D women with non-significantly higher levels in GDM women than other women. These findings indicated the presence of an association between pregnancy and diabetes and this inflammatory adipocytokine, irrespective of being pathogenic or resultant relation. Similarly, multiple previous and recent studies detected higher levels of inflammatory biomarkers in GDM women than in women who had normal

pregnancies [23, 29]. A recent study attributed the relation between high blood glucose and inflammatory markers to the high incidence of small intestinal bacterial overgrowth that may increase maternal blood glucose by affecting inflammatory response [30].

At-enrolment plasma lipids at-enrolment were significantly higher in women going to have GDM than non-GDM and NP/ND women, and statistical analyses defined high TC levels as a predictor for oncoming GDM with a high specificity rate. Similarly, Cibickova et al. [31] detected high TG and non-HDL levels in women who had high BG on 75-OGTT in the 2nd trimester than in non-GDM pregnant women. Also, Yang et al. [32] detected a relationship between constituents of metabolic syndrome and pregnancy complicated by GDM and found TC levels can predict this complication. Moreover, Zhong et al. [33] using lipidomics analyses found significant associations between dysregulated lipids concentrations and maternal glucose. Multiple trials were conducted to explore the relationship between high lipid and glucose measures during pregnancy; Jiang et al. [34] detected significant downregulation of glycerolphospholipid metabolism in GDM women with a negative correlation between phosphatidyl-choline and –ethanolamine levels and maternal BG concentration, while the correlation was positive with triacylglycerol levels. Further, Luo et al. [35] reported a positive relationship between levels of several key lipid metabolites as γ-linolenic acid, and heptadecanoic acid and BG levels in GDM, while Attique et al. [36] attributed the coincidence of GDM and dyslipidemia in pregnant women to the disturbing levels of neuregulin-4.

Conclusion

GDM may be related to the interplay between high levels of VEGF, inflammatory cytokines, and hyperlipidemia. High blood levels of VEGF and TC can discriminate against women vulnerable to developing GDM with high sensitivity, specificity, and negative predictive values.

Recommendations

Estimation of blood levels of lipid and VEGF before pregnancy is recommended to be applied in health awareness programs to allow time for procedures applied to reduce these levels if were found to be high.

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