

# Altered Expression of Angiogenic Biomarkers in Pregnancy Associated with Gestational Diabetes

Ebtisam Al-Ofi  
Aziza Alrafiah   
Salman Maida  
Safa Almaghrabi  
Nora Hakami

<sup>1</sup>Department of Physiology, Faculty of Medicine, King AbdulAziz University, Jeddah, Saudi Arabia; <sup>2</sup>Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King AbdulAziz University, Jeddah, Saudi Arabia

**Background:** Gestational diabetes mellitus (GDM) typically occurs during the third trimester of pregnancy. Maternal hyperglycemic may influence the expression of pro-and anti-angiogenic factors. Altered levels of angiogenic biomarkers in GDM pregnant women are associated with abnormal placentation. This study aimed to investigate the rates of expression of five angiogenic biomarkers called vascular endothelial growth factor-A (VEGF-A), angiopoietin-2, endoglin, endothelin-1, and granulocyte colony-stimulating factor (G-CSF) in GDM.

**Methods:** The samples were obtained from normal (n=9) and GDM (n=10) pregnancies. Multiplex assay was used to assess the levels of angiogenic biomarkers including VEGF-A, endoglin, endothelin-1, angiopoietin-2, and G-CSF in serum samples. All data were statistically analyzed using an unpaired Student's *t*-test. Correlations between measured parameters were made using Pearson correlations.

**Results:** VEGF-A, endoglin, endothelin-1, and angiopoietin-2 levels in GDM were significantly higher (P value = 0.001, 0.042, 0.049, 0.001; respectively) compared to control. However, G-CSF level exhibited a non-significant increase (P=0.466) in GDM compared to healthy controls. There was a significant positive correlation between angiopoietin-2 with endoglin, endothelin-1, and VEGF-A. Moreover, there was a significant positive correlation between VEGF-A with endoglin and endothelin-1. Most interestingly, there was a significant positive correlation between G-CSF with endothelin-1.

**Conclusion:** The angiogenic biomarkers were highly altered in pregnant women with GDM. The study provides a novel advance in the field of gestational diabetes, in terms of increase of angiogenic factors that can modify the vascularization of the placenta, the development of fetal vascular system and the insulin resistance itself.

**Keywords:** gestational diabetes mellitus, vascular endothelial growth factor A, angiopoietins, endoglin, endothelin-1, granulocyte colony-stimulating factor, pre-eclampsia

## Introduction

Gestational diabetes mellitus (GDM) is described as glucose intolerance that manifests clinically in the second half of pregnancy. Approximately one in ten pregnant women worldwide are affected by GDM.<sup>1</sup> It is associated with the placental vascular tree's morphological changes: increased surface area and capillary branching pregnancy.<sup>2</sup> So far, maternal hyperglycemia has been regarded as a significant mechanism for GDM-associated placental vascular alterations. Moreover, maternal hyperglycemic, pro-inflammatory conditions may also influence the expression of pro-and anti-angiogenic cytokines and growth factors.<sup>3</sup>

Correspondence: Aziza Alrafiah  
Department of Medical Laboratory  
Technology, Faculty of Applied Medical  
Sciences, King AbdulAziz University, PO  
Box 80200, Jeddah, 21589, Saudi Arabia  
Tel +966 126401000 Ext. 23495  
Fax +966 126401000 Ext. 21686  
Email aalrafiah@kau.edu.sa

Angiogenesis is a physiological process that involves forming new blood vessels from the existing one in several steps, including vascular sprouting, tubule morphogenesis, and stabilization and adaption of the vessel.<sup>4</sup> Hence, every step of the angiogenesis process targets regulation by paracrine, autocrine, and environmental factors.<sup>5</sup> Feto-placental angiogenesis and vascular development are tightly regulated by the interaction between pro- and anti-angiogenic factors. A branched vascular network is essential for the placental formation and depends on these factors, including the vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), soluble endoglin (sEng), endothelin-1 (ET-1), and granulocyte colony-stimulating factor (G-CSF).<sup>6</sup> Abnormal vasculature in the placenta is considered the most common placental pathology found in many pregnancy complications.<sup>7</sup> Expression of the previous angiogenic biomarkers assists in vascular development and promotes angiogenesis for proper fetal development, regeneration, and wound healing.<sup>8</sup>

VEGF promotes angiogenesis in embryonic development and is essential in adults' wound healing.<sup>9</sup> Any disruption in VEGF expression may lead to lethal outcomes resulting from abnormal blood vessel formation during embryogenesis.<sup>10</sup> Nevertheless, Ang-2 may have a pro- or anti-angiogenic effect, depending on the presence of VEGF.<sup>11</sup> In the absence of VEGF, Ang-2 activates endothelial cell apoptosis, vessel regression, and inhibits angiogenesis. However, in the presence of VEGF, Ang-2 works as an antagonist of Ang-1, destabilizes the interaction between endothelial cells and the supporting cells, induces vessel instability and the formation of disorganized and immature new blood vessels.<sup>12</sup> Yet, endoglin is an anti-angiogenic molecule that has a significant role in pregnancy complications.<sup>13</sup> Increased placental gene expression of ENG may lead to vascular dysfunction causing chronic fetal hypoxia, which may activate VEGF to increase angiogenesis as a feedback response to restore the fetus's placental circulation.<sup>14</sup> In contrast to, stimulation of endothelin-1 A receptor via ET-1 that is accountable for vasoconstriction, activation of its endothelin-1 B receptor leads to vasodilation by enhanced nitric oxide development to meet the plasma volume expansion necessary for good pregnancy outcomes. However, increased stimulation or malfunction of the ET system in pregnant women is reported as resulting in hypertension leading to

preeclampsia.<sup>15</sup> In type 2 diabetes mellitus patients (T2DM), plasma levels of ET-1 are increasingly contributing to diabetes pathophysiology,<sup>16</sup> which is ET-1 causes insulin resistance via direct action on the skeletal muscle rather than reducing insulin delivery resulting from vasoconstriction.<sup>15</sup> Moreover, G-CSF plays a crucial role in pregnancy success by reducing pregnancy loss via activating the ovarian function and embryo implantation and promoting endometrial thickening.<sup>17</sup> It is demonstrated that G-CSF: G-CSFR (specific receptor) interaction increases the secretion of VEGF, a factor that stimulates placental blood vessel formation.<sup>17,18</sup>

Alteration in paracrine angiogenic biomarkers may affect the feto-placental endothelium and may lead to gestational diabetes mellitus, and the subsequent maternal and neonatal complications, which include a high risk of developing preeclampsia, recurrent miscarriage, retinopathy, and an increase in infant birth weight. These, in turn, may contribute to GDM related changes in placental angiogenesis.<sup>2,3,19</sup> Therefore, we hypothesized that angiogenic biomarkers could be altered in women with GDM and perhaps lead to maternal and neonatal complications and morbidities. To test this hypothesis, we compared the serum levels of five fundamental angiogenic biomarkers called VEGF, angiopoietin-2, endoglin, endothelin-1, and G-CSF in GDM and healthy pregnant women.

## Materials and Methods

### Ethical Approval

The study was approved by the ethical committee of King Abdulaziz University Hospital (Reference No 336–16). Informed written consent was obtained from all participants. This study was conducted in accordance with the Declaration of Helsinki.

### Materials

The Angiogenesis/Growth Factor Panel 1 Antibody-Immobilized Magnetic Beads kits (Anti-Human Angiopoietin-2 Bead (CatNo. HANGPT2-MAG), Anti-Human Endoglin Bead (CatNo. HENDGLN-MAG), Anti-Human Endothelin-1 Bead (CatNo. HET1-MAG), Anti-Human G-CSF Bead (CatNo. HAGGCSF-MAG), and Anti-Human VEGF-A Bead (CatNo. HVEGF-MAG) were purchased from Millipore Corp (USA). The standards (CatNo. HNDG2-8036-2), quality

controls 1 and 2 (CatNo. HNDG2-6036-2), bead diluent (CatNo. LBD), detection antibodies (CatNo. HNDG2-1036-2), and streptavidin-phycoerythrin (CatNo. L-SAPE6) were purchased from Invitrogen.

## Sample Collection and Processing

Venous whole blood was collected by venipuncture from nine healthy pregnant women (aged 20–40 years) with no previously or currently diagnosed GDM and ten pregnant women with GDM (aged 20–40 years). GDM was diagnosed on an oral glucose tolerance test of 75 g following the current IADPSG guidelines.<sup>20</sup> Subjects with bleeding disorders, coagulation disorders, or those who are on anticoagulant therapy were excluded.

Blood was collected into plain tubes containing no anticoagulants. The blood was allowed to clot for at least 30 minutes before centrifugation for 10 minutes at 1000×g to obtain serum, which was aliquoted and stored at –80°C to be used later in the determination of angiogenic biomarkers concentrations.<sup>21</sup> The procedure of measuring angiogenic biomarkers is based on antigen-antibody reaction on immunoassay machines called Luminex 200 (Luminex 100/200, USA).

## Methods

The angiogenic biomarkers concentrations in the serum were measured using multiplex xMAP technology on a Luminex 200 instrument with commercially available panels from Millipore Corporation (Billerica, MA, USA). The procedures were performed according to the manufacturer's instructions, and the control samples that were provided within the kits were assayed in each analysis, and samples were run in duplicate. In our study, the levels of VEGF-A, endoglin, endothelin-1, angiopoietin-2, and G-CSF were studied. The data were generated by the 3.1 Xponent software package (Luminex Corporation, USA).

## Statistical Analysis

The data were analyzed using version 7 GraphPad Prism software. All data were statistically analyzed using an unpaired Student's *t*-test. Correlations between measured parameters were made using Pearson correlations. *P* – values less than 0.05 were considered significant. Figures were generated using GraphPad Prism (version 7; San Diego, USA).

## Results

### Clinical Characteristics of Pregnant Women Participating in the Present Study

All participants in the present study were recruited from KAUH, at their routine antenatal clinic before any diabetic therapy was administered, the regardless diagnosis was confirmed later, and they were distributed into the GDM group or healthy pregnant group. Maternal age, gestational age (weeks), and other gestational-related data were collected from ten GDM women and compared to nine normal pregnant (NP) women. Clinical characteristics of the study participants are presented in Table 1. The study participants' clinical characteristics, including age, gestational age at GDM diagnosis, gravidity, parity, abortion, intrauterine fetal death, and body mass index, were non-significantly different between GDM groups and the healthy participants.

### Comparison of Angiopoietin-2 (Pg/MI) Levels Between Control Pregnant and GDM-Samples

Angiopoietin-2 serum levels in GDM women were significantly higher compared to controls healthy pregnant women (healthy vs GDM; 3769.67±374.19 pg/mL vs 15,343.90 ±807.90 pg/mL, *P* = 0.001) as shown in (Figure 1).

**Table 1** Clinical Characteristics of Pregnant Women Participating in the Present Study

Variables	GDM (n = 10)	NP (n = 9)	P-value
Age (years)	32.7 ± 7.1	33 ± 8	NS
Gestational age (weeks)	26.4 ± 2.7	25.5 ± 1.8	NS
Gravidity	4.8 ± 3.1	4.6 ± 3.6	NS
Parity	2.1 ± 1.7	3.7 ± 2	NS
Abortion or IUFD	2.7 ± 1.4	0.9 ± 1.6	NS
BMI	30.5 ± 5	28 ± 5.7	NS
RBS	7.8 ± 2.5	4.6 ± 0.7	0.013
GTT			
Fasting	5.6 ± 0.8	-	
2hrs	9.8 ± 2.5	-	

**Abbreviations:** BMI, body mass index; GDM, gestational diabetes mellitus; IUFD, intrauterine fetal death; NS, not significant; RBS, random blood sugar; GTT, glucose tolerance test.

## Comparison of VEGF-A (Pg/MI) Levels Between Control Pregnant and GDM-Samples

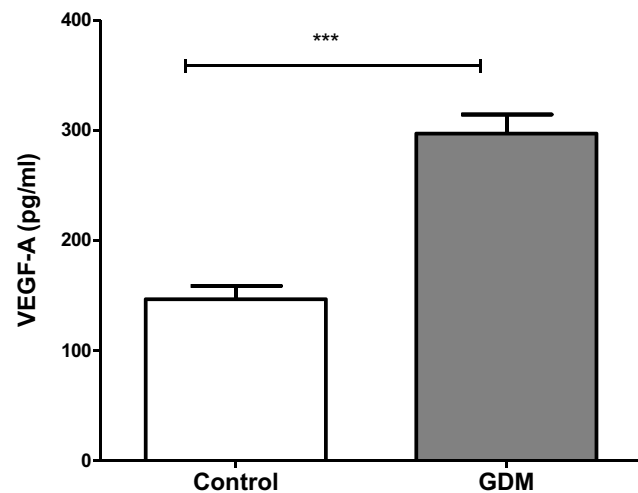
The serum level of VEGF-A in the GDM group was significantly higher ( $P = 0.001$ ) compared to the healthy controls (healthy vs GDM;  $146.60 \pm 12.03$  pg/mL vs  $296.92 \pm 17.37$  pg/mL,  $P = 0.001$ ) (Figure 2).

## Comparison of Endoglin (Pg/MI) Levels Between Control Pregnant and GDM-Samples

Furthermore, the difference in endoglin concentration in serum between GDM women and healthy participants was significant (healthy vs GDM;  $1444.78 \pm 82.30$  pg/ mL vs  $1814.06 \pm 141.10$  pg/mL,  $P = 0.042$ ) (Figure 3).

## Comparison of Endothelin (Pg/MI) Levels Between Control Pregnant and GDM-Samples

Endothelin-1 concentration in the GDM participants ( $6.58 \pm 0.13$  pg/mL) was significantly higher ( $P = 0.049$ ) in comparison to the healthy group endothelin-1 level ( $6.12 \pm 0.17$  pg/ mL) (Figure 4). However, G-CSF serum level non significantly ( $P=0.466$ ) increased in the GDM group (healthy vs GDM;  $124.61 \pm 2.12$  pg/ mL vs  $129.46 \pm 5.86$  pg/mL), but to

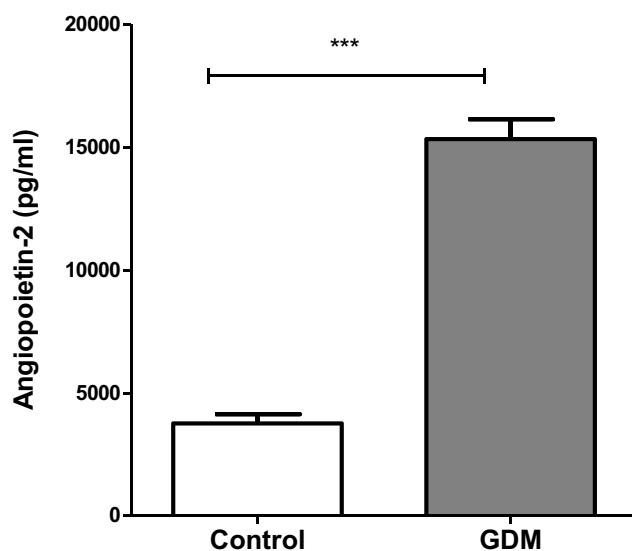


**Figure 2** Comparison of VEGF-A (pg/mL) levels between control pregnant and GDM-samples. Data are expressed as mean  $\pm$  standard error. \*\*\* $P = 0.001$  compared to control.

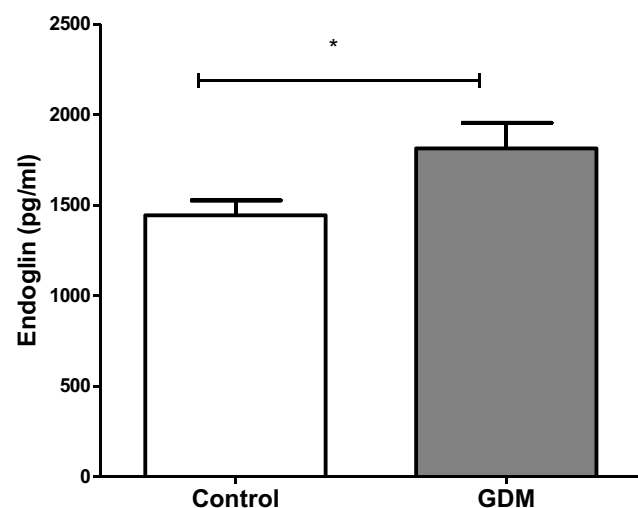
a lesser extent compares to the rest of the angiogenic biomarkers.

## Correlations Between Angiotensin-2 and Endoglin in Control

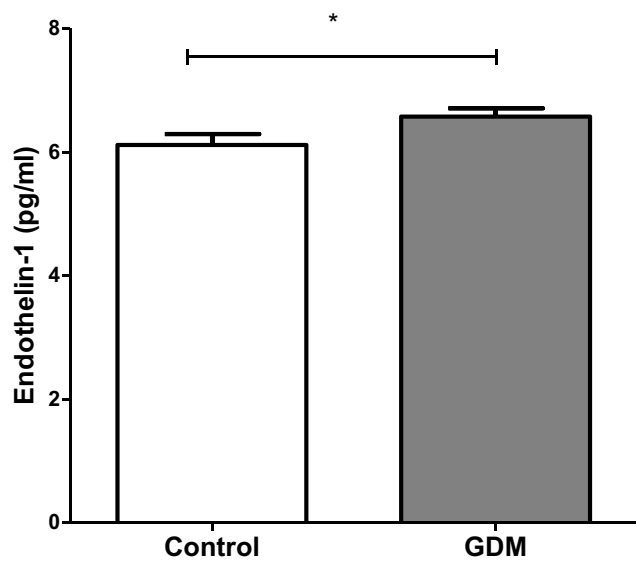
In the control group, only angiotensin-2 exhibited a significant positive correlation with endoglin, using the Pearson product-moment correlation test (Figure 5). However, the other studied biomarkers did not demonstrate significant correlations within the control group (data unshown).



**Figure 1** Comparison of angiotensin-2 (pg/mL) levels between control pregnant and GDM-samples. Data are expressed as mean  $\pm$  standard error. \*\*\* $P = 0.001$  compared to control.



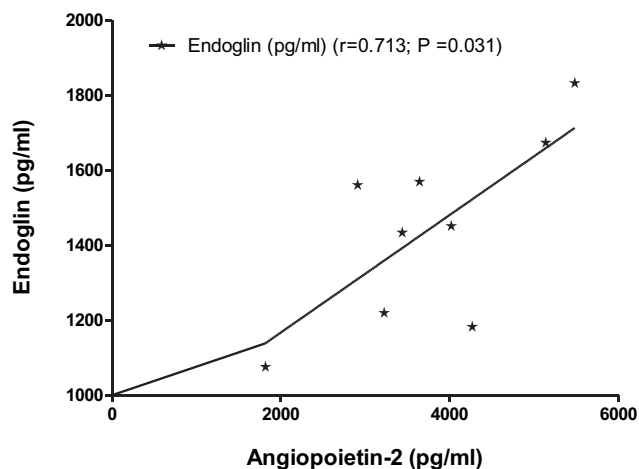
**Figure 3** Comparison of endoglin (pg/mL) levels between control pregnant and GDM-samples. Data are expressed as mean  $\pm$  standard error. \* $P < 0.05$  compared to control.



**Figure 4** Comparison of endothelin (pg/mL) levels between control pregnant and GDM-samples. Data are expressed as mean  $\pm$  standard error. \*P < 0.05 compared to control.

### Correlations Between Angiotensin-2 with Endoglin, Endothelin-1, and VEGF-A in GDM Patients

In the GDM patients: endoglin, endothelin-1 and VEGF-A showed significant positive correlations ( $r= 0.868$ ,  $P=0.001$ ), ( $r= 0.729$ ,  $P=0.017$ ) and ( $r= 0.964$ ,  $P=0.001$ ), respectively with serum level parameter of angiotensin-2 (Figure 6).



**Figure 5** Correlations between endoglin and angiotensin -2 in control group. The correlation coefficient was made using the Person test.

### Correlations Between G-CSF and Endothelin-1 in GDM Patients

Despite of a reciprocal small sample size, the G-CSF exhibited significant positive correlations with the endothelin-1 parameter ( $r= 0.667$ ,  $P=0.035$ ) (Figure 7). Interestingly, endoglin and endothelin-1 exhibited positive significant correlations ( $r= 0.729$ ,  $P=0.017$ ) and ( $r= 0.706$ ,  $P=0.023$ ); respectively with the VEGF-A (Figure 8).

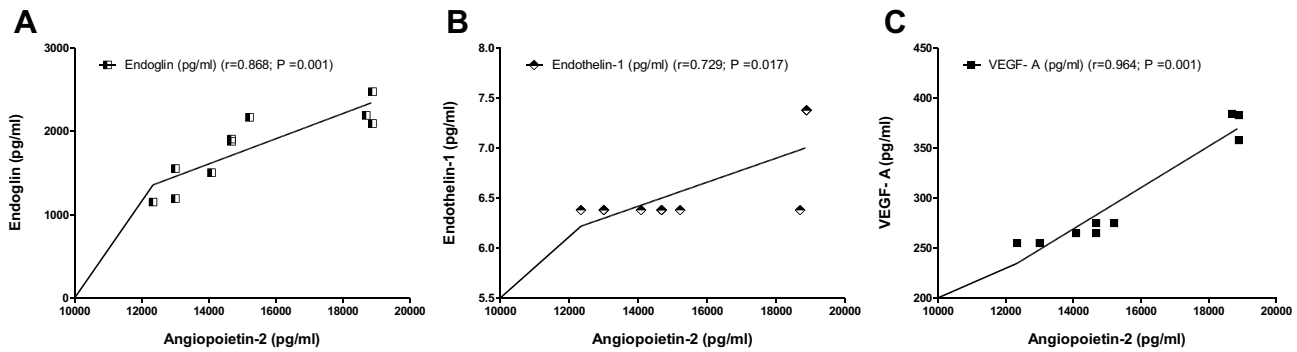
### Discussion

The current study demonstrated that the serum level of angiogenic biomarkers (VEGF, angiotensin-2, endoglin, and endothelin-1) in pregnant women with GDM were significantly higher compared to healthy pregnant women. However, G-CSF level was high in GDM patients, but the result was not significant compared to normal participants.

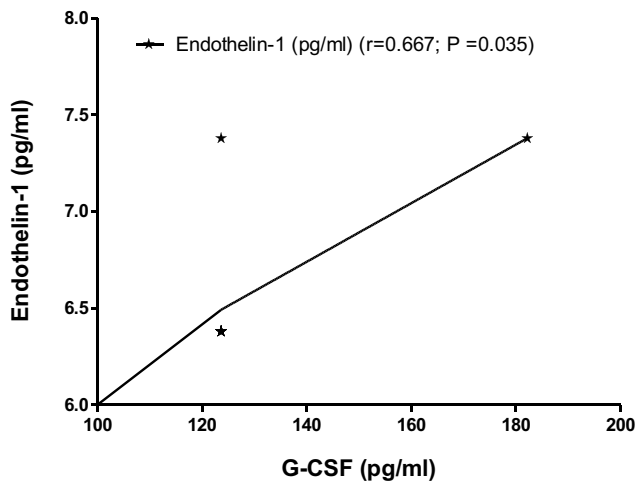
Pathological changes in maternal blood properties (such as hypoxia or hyperglycemia); or pro-and anti-angiogenic factors such as VEGFs and soluble endoglin may directly affect the growth and function of fetoplacental vessels. Alterations in the fetoplacental vessels may be associated with the pathogenesis of GDM.

Expression of the angiogenic biomarkers helps in vascular development and promotes angiogenesis for proper fetal development.<sup>8</sup> Any alteration in angiogenic biomarkers' levels is associated with abnormal placentation and the subsequent maternal and neonatal complications.<sup>19</sup>

In the present study, the GDM group showed a significant increase in serum VEGF-A levels than healthy pregnant women. The results presented here are similar to those of another recent study that investigated GDM rats and showed a significant increase in serum VEGF-A level and advanced glycation end-products (AGEs) concentrations.<sup>22</sup> The potential mechanism(s) for increase VEGF-A level might be that AGEs perform their harmful effects, whether by actively destroying cells or by binding to the receptor of advanced glycation end-products (RAGE). As binding AGEs with RAGE enhances activation of the NF- $\kappa$ B pathway and increases VEGF production, rendering this significant mechanism underlying malfunction at the placental barrier of GDM.<sup>23</sup> Another potential mechanism is that elevated maternal hemoglobin A1c (HbA1c) levels may result in a discrepancy in supply and demand for oxygen, resulting in a temporary decrease in fetal oxygen levels (ie, hypoxia). Transient fetal hypoxia can cause increased placental synthesis and VEGF release, as well as VEGF receptor expression.<sup>1</sup>



**Figure 6** Correlations between angiopoietin –2 and endoglin (A), angiopoietin –2 and endothelin-1 (B) and angiopoietin –2 and VEGF- A (C) in GDM patients. Correlation coefficient was made using Pearson test.



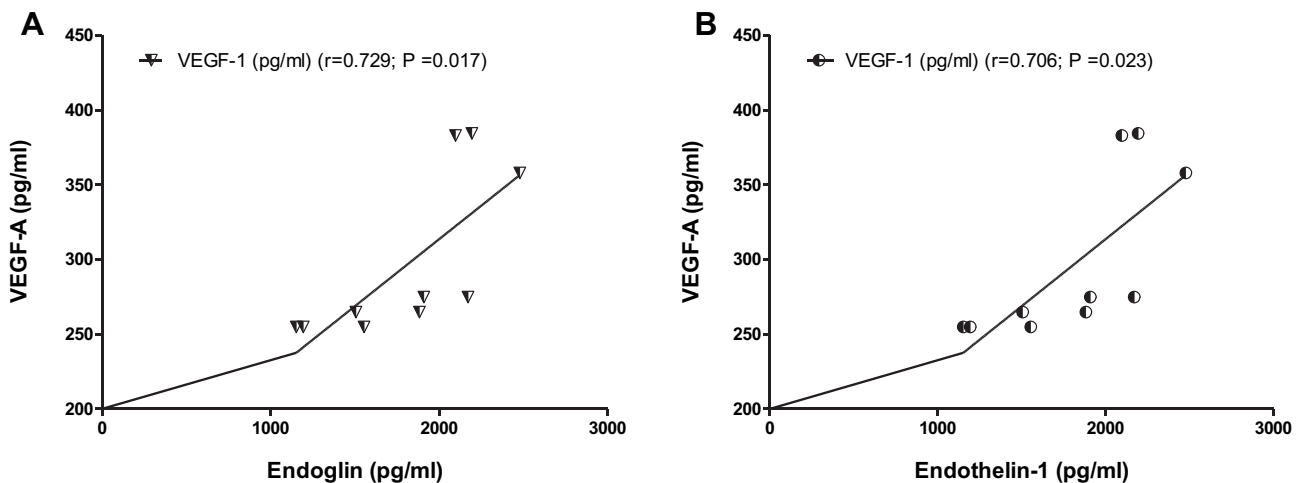
**Figure 7** Correlations between G-CSF and endothelin-1 in GDM patients. The correlation coefficient was made using the Pearson test.

However, our results contrast to another study that demonstrated decreased concentrations of VEGF-A and

VEGF receptor in placental tissues collected from GDM females compared to healthy pregnant women.<sup>24</sup> The explanation for these conflicting reports may be due to differences in the sample as Meng et al (2016) used placental tissue samples while in our study, we measured the serum level of VEGF-A. The discrepancy between the VEGF-A concentration between placental tissues and serum could be a pathogenic disease feature.

Furthermore, we have found that Angiopoietin-2 serum concentrations significantly increased in GDM patients compared to the healthy group. Similar results were observed in previous research and reported a significant positive correlation between angiopoietin with insulin resistance in the GDM group and concluded that serum angiopoietin level could be a predictor of GDM.<sup>25</sup>

In particular, the VEGF-A mechanism of action tends to be similar to the angiopoietin-2, especially in the late stage of angiogenesis. They act through distinct tyrosine kinase receptors, which indicates that VEGF-A has



**Figure 8** Correlations between endoglin and VEGF-A (A) and endothelin-1 and VEGF-A (B) in GDM patients. The correlation coefficient was made using the Pearson test.

indirectly affected the angiopoietins functions by the proteolytic shedding of the angiopoietin receptor.<sup>26,27</sup> That could explain our findings that there was a significant positive correlation between the angiopoietin serum level and VEGF in the GDM group.

In the current study, the GDM group showed a significant increase in endoglin serum concentrations than the healthy pregnant group. A similar finding was shown in pregnant women with T1DM relative to those of non-diabetic women, which may lead to the raised prevalence of preeclampsia and interfere with the development of blood vessel complications.<sup>28</sup> Additionally, we have found that serum level of VEGF-A significant correlations endoglin, which is in agreement with Masuyama et al (2007) study.<sup>29</sup>

Moreover, the endothelin-1 serum concentrations were significantly higher in the GDM pregnant participants. Similar results were reported in patients with T1DM and T2DM, and it has been suggested that changes in plasma endothelin-1 levels may precede vascular complications associated with hypertension and diabetes.<sup>30</sup> It has been found the human placenta expresses endothelin-1, and its circulating levels are high in pregnancies suffering from GDM.<sup>31</sup> In contrast, two previous studies reported a normal endothelin-1 plasma level in women with GDM.<sup>32,33</sup>

In the present study, the GDM group showed a non-significant increase in G-CSF serum concentrations than the healthy control group. Previous research showed similar results that the serum G-CSF rates among diabetes subjects were significantly higher than those without diabetes; also the diabetic group that used insulin and oral hypoglycemic agents, their serum G-CSF levels were lower.<sup>34</sup> However, our results contrast to another study that G-CSF level decreased in patients with T1DM or T2DM compared to control.<sup>35</sup> G-CSF can play a significant role in stimulating vascular endothelium injury and granulocytes, which may play a significant role in the development of preeclampsia.<sup>36</sup> That effect could explain the significant positive correlation in the current study between the G-CSF and the endothelin-1 as the endothelin-1, known as a potent vasoconstrictor. It could interfere with the normal angiogenesis function of diabetic mothers by blocking off some angiogenic biomarkers such as G-CSF.

## Conclusion

The present study showed that in GDM pregnant women, the level of these factors VEGF, angiopoietin-2, endoglin,

and endothelin-1 are significantly higher than in control. Further research is warranted to understanding the association and interaction between altered levels of angiogenic factors and pregnancy associated with gestational diabetes and investigated their suitability for therapeutic and diagnostic applications.

## Institutional Review Board Statement

The study was conducted according to the guidelines of the bioethics and research committee of the King Abdulaziz University (KAUH), Jeddah, Saudi Arabia, and approved by the Institutional Scientific and Research Committee under the reference number of (Reference No 336-16).

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## Disclosure

The authors declare no conflicts of interest for this work.

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