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Histopathological and Immunohistochemical Changes in Placentas of Women with Gestational Diabetes Mellitus: CD56 and VEGF Expression

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ABSTRACT:

Background: Gestational diabetes mellitus (GDM) is an increasingly prevalent metabolic disorder that can adversely affect both maternal and neonatal outcomes. The placenta is a key target organ in GDM, where metabolic and vascular disturbances may cause structural alterations and altered expression of angiogenic and immune-related markers. This study evaluated placental histopathological changes and the immunohistochemical expression of vascular endothelial growth factor (VEGF) and CD56 in pregnancies complicated by GDM.

Methods: This prospective comparative study included 120 pregnant women: 60 with GDM and 60 normoglycemic controls. GDM was diagnosed at 24–28 weeks of gestation. Maternal age, gestational age, parity, placental weight, and placental diameter were recorded. Placental specimens underwent routine histopathological examination and immunohistochemical staining for VEGF and CD56.

Results: Placentas from the GDM group showed multiple histopathological alterations, including villous overcrowding, decreased terminal-villus density, increased immature intermediate villi and terminal-villus size, increased syncytial knots, thickening of the villous basement membrane and villous vessels, cytotrophoblastic prominence, fibrinoid deposition and necrosis, fibrosis, increased Hofbauer cells, calcification, chorangiosis, fetal vascular thrombosis, nucleated fetal red blood cells, and villous edema. VEGF expression was significantly higher in GDM placentas than in controls, with moderate-to-strong immunoreactivity in syncytiotrophoblasts, villous stromal cells, and endothelial cells. In contrast, CD56 expression showed no statistically significant difference between the GDM and control groups.

Conclusion: GDM is associated with substantial placental histopathological alterations and increased placental VEGF expression, indicating disturbed placental vascular adaptation and angiogenesis. CD56 immunoexpression was not significantly altered in the placental tissue examined. Further studies that include the decidua basalis or placental bed and assess earlier gestational ages may better define the role of CD56-positive uterine natural killer cells in GDM. CD56-positive uterine NK cells are especially prominent in

early decidua and participate in spiral-artery remodeling, which helps explain why they may be limited in term villous placental samples.

INTRODUCTION

The placenta is a highly specialized and complex organ that plays essential roles in maintaining pregnancy and supporting fetal growth. It serves as the fetus kidneys, liver, stomach, and lungs throughout pregnancy. Additionally, the placenta has important endocrine functions that regulate the mother's physiology and metabolism and offer a secure environment for the fetus to grow [1, 2].

Insulin-dependent glucose absorption is further reduced in the maternal tissues as GDM progresses, leading to the development of hyperglycemia. Increased placental transfer of glucose and fetal hyperglycemia results from concentration-dependent placental transfer of glucose under conditions of maternal hyperglycemia and placental normal function. Hyperinsulinism develops because of these changes. The newborn's residual hyperinsulinism raises the risk of hypoglycemia if the umbilical supply of glucose is abruptly stopped following delivery [3].

Increased fibrinoid material, villous edema, and thickening of the basement membrane in trophoblastic cells have all been linked to gestational diabetes histopathologically [4]. GDM placentae have been shown to have thicker endothelial basement membranes and more collagen fibers in the villous stroma [5]. The physical and histological changes in placentae with gestational diabetes, however, are variable and even somewhat debatable Placenta [6].

The assessment of CD56 expression in placental tissue may provide additional information regarding immune related changes associated with GDM, whereas interpretation of CD56 immunoreactivity in term villous tissue should take into consideration the gestational distribution and localization of uterine NK cells. Alteration in the distribution and functional activity of CD56 positive uterine NK cells have been associated with abnormalities in placentation and spiral artery remodeling [7,8].

Vascular endothelial growth factor (VEGF), a central regulator of angiogenesis, is essential for villous vascularization and fetal oxygenation. Dysregulation of VEGF has been associated with villous immaturity, chorangiosis, and maternal vascular malperfusion[2,9].

MATERIALS AND METHODS

this cross-sectional comparative study included 120 singleton pregnant women, among them, 60 pregnant women diagnosed with GDM between 24-28 weeks of gestation and 60 normoglycemic women who had no history of GDM or any complications in their current or previous pregnancies were included (as control).

Fresh Placental specimens were collected from women with GDM and normoglycemic women at Department of Obstetrics and Gynaecology at Al-Imamain Al-Kadhimiyain (AS) Medical city, Al-Alawiya Teaching Maternity Hospital, Ibn Al-Baladi Hospital and Dowlay private hospital during the period from January 2025 to January 2026. According to the predefined inclusion criteria which include gestational age of more than 24 weeks. Personal, obstetric history, family history for diabetes mellitus, gestational diabetes period and predisposing factors with previous pregnancies were assessed.

For histopathological analysis, placentae were dissected and sampled. From the chosen placental lobule, two standard samples were obtained: one from the center region and one from the periphery. A clean, labeled plastic jar with 10% NBF solution was filled with fresh placental tissue pieces [10].

Standard preparation of histopathological tissues

Following delivery, placental specimens were transferred to the laboratory for histopathological processing. The tissue samples were cut into small pieces and processed according to standard histopathological procedures described by Suvarna etal. [11]. Tissue samples were fixed by immersing them in a formalin fixative solution10% for 24 hours. The tissues were then dehydrated by passing them through increasing alcohol series (70%, 80%, 90%, and 100% ethyl alcohol). The specimens were cleared in xylene three times with each step lasting 30 minutes. The tissues were embedded in paraffin blocks after being incubated twice for forty-five minutes at 58°C in paraffin wax. Using a microtome, four μm thickness pieces of the blocks were placed on three separate ordinary slides for histochemical staining with Hematoxylin and eosin (H&E), Masson's trichrome staining was

performed to evaluate (Villous stroma, Basement membrane thickness, Syncytial knots, Vessel walls thickness and fibrosis). according to Bancroft, et.al. (2013).[12].

A compound light microscope (Meijitechno, Japan) equipped with a digital camera (Canon, Japan, 18 megapixels) was used to analyze the sections. The histological sections were evaluated with the assistance of an experienced pathologist, images were independently examined using a Multihead teaching microscope (Genex, USA).

- **Immunohistochemical staining tissue**

Deparaffinization was first carried out in xylene. Following immersion in absolute alcohol at decreasing concentrations of 100%, 96%, and 70%, the specimens were washed with distilled water. Antigen retrieval was performed by incubating the sections for 30 minutes at room temperatures, Specimens were first rinsed with PBS buffer after this process, then rinsed once more with PBS buffer after being treated in H2O2 for five minutes to block endogenous peroxidase activity. A solution of the primary monoclonal antibody was then applied to the specimens. CD56 (clone 123C3, Catalog No: IR628, Dako Denmark), VEGF (clone VG1, Code No: M7273, Dako Denmark) WAS solution was eventually used to wash the specimens. Specimens were initially submerged in post-primary solutions to detect immunohistochemistry staining. Following washing, the specimens were submerged in Polymer solution and subsequently in DAB solution, a chromogen. The specimens were then stained with hematoxylin and eosin. After the automatic processor completed the earlier steps, the specimens were rinsed with tap water and dehydrated using increasing densities of xylene and ethanol solution (70, 90, and 100% v/v in that order). A glass slide was treated with two drops of DPX, covered with a cover slip, and inspected.

For each of the two antibodies that were investigated in this investigation, the previously described immunohistochemical staining technique was performed. While the monoclonal antibody VEGF required a dilution of 1:25 to 1:50, the monoclonal antibody CD56 was already prepared and ready to use.

The Immunohistochemical staining intensity was classified as negative (-), weak (+), moderate (++) and strong (+++). The outcomes were then statistically examined to determine their significance. Means or percentages were used to display the data. $P < 0.05$ results were deemed statistically significant.

RESULTS AND DISCUSSION

Diabetes is a metabolic disease that affects every organ in the body, including temporary organs like the placenta. As a result, it can have a negative impact on neonatal outcomes and is therefore a major worry [13,14]. Numerous histological differences were identified in the current study after placental sections were examined under a light microscope. These variations are listed as follows:

Villi crowding (Fig. 1-A):

In the current investigation, villous crowding was more frequently observed in the GDM group than in the control group. [6,15,16,17] found that diabetic placentas were more likely to have histological abnormalities including villi crowding, which was consistent with our findings.

An increase in the number of immature intermediate villi (Fig.1-B):

An individual risk of stillbirths has been linked to a placental anomaly [18]. An increased proportion of immature intermediate villi may reflect delayed villous maturation and has been associated with chronic fetal hypoxia[16;19], as defects in placental maturation have been linked to chronic fetal hypoxia [20;17].

Reduced density of terminal villi (Fig. 1-B):

An apparent increase in terminal villous density may result from an increase in villous size, particularly when immature intermediate and terminal villi are enlarged [21]. Numerous investigations found a correlation between the prevalence of immature villi and inadequate or nonexistent terminal villi in GDM [16; 22;23;24].

Terminal villi size increase (Fig. 1-B):

In comparison to controls, terminal villous size dramatically increased in diabetes group in the current study. According to Mayhew (2002), terminal villi size differed significantly between diabetes and control placentae [2;6;25].

Focal syncytial knots. (Fig. 1-C):

Syncytial knotting, also known as Tenny-Parker changes, refers to an increase in the number of syncytial knots, bridges, and shoots [26]. In a previous study, Gheorman et al. (2012) found that placentae from pregnant women with diabetes had more syncytial knots than the typical placenta [21;27].

Terminal villi's cytotrophoblast (Fig. 1-D):

Increased cytotrophoblastic prominence was observed in the GDM group. This finding is consistent with previous reports describing delayed villous maturation and increased cytotrophoblast persistence in diabetic placentas [28]. According to Bentley-Lewis et al. (2014), placental abnormalities in GDM include elevated cytotrophoblastic [6;29].

Thickness of the vasculo-syncytial membrane (Fig. 1-D):

The weakening of the vascular-syncytial membrane lowers the barrier between maternal and fetal circulation. This may have an adverse effect on oxygen distribution, metabolism, and transplacental transport [16;30]. The improved risk of intrauterine losses in diabetes women may be caused by delayed villous maturation, which could lead to decreased vasculo-syncytial formation [23;31].

Between the terminal villi, an extravillous fibrinoid (Fig. 1- E)

Increased extravillous fibrinoid deposits are indicative of pathological conditions and are often at odds with normal fetal growth [16;32].

Fibrinoid in the stem and terminal villi (Fig. 1-F)

Increased stromal fibrosis in the stem villi is considered pathological. Due to inadequate uptake by the fetal capillaries, diabetic women have elevated villous stromal oxygen partial pressure, which stimulates collagen formation [24]. [25] found greater villous fibrosis in GDM placentas under insulin control, but this finding was not detected in GDM placentas under diet control or in control women [22; 26;33].

Stem and terminal villi fibrinoid necrosis (Fig. 1-F):

Within the villi, fibrinoid necrosis was identified as non-cellular homogeneous eosinophilic material. In certain areas, the basement membrane and the entire villous stroma were compressed by the increased fibrinoid material [26]. When compared to the control placenta, histological abnormalities such fibrinoid necrosis were found more frequently in GDM [16;27;31] placentae.

The stroma has a decent cell population, the stem villi have an indented border, and the trophoblastic layer continues (Fig. 1-G): According to Dubova et al. (2011), the GDM group had a higher trophoblastic layer and cell population than the control group, which is consistent with our findings [18].

Immature intermediate villi with a higher density of Hofbauer cells and a loose reticular stroma (Fig. 1-G):

An increased density of Hofbauer cells was observed within immature intermediate villi in the GDM group. Hofbauer cells are fetal macrophages located within the villous stroma and may be involved in placental immune and vascular responses, but these women also have the best metabolic control [30;34].

Both external and intracellular calcification (Fig. 1-H):

In numerous investigations, calcifications were seen in the placentae of the GDM group as both extracellular and intracellular basophilic deposits after being stained with hematoxylin and eosin [2;35].

Chorangiosis (Fig. 1-I):

The increased frequency of chorangiosis observed in GDM placentas may reflect a compensatory response to altered oxygenation and increased angiogenic demand. The concomitant increase in VEGF expression observed in the present study may support a role for altered angiogenic signaling in these vascular changes [36;37].

Villi vessel thickening (Fig.1-I)

thickness of the basement membrane and endothelial proliferation in the villi vessel walls. Additionally, it has been noted that elevated blood glucose levels trigger oxidative stress (OS) and subsequent changes in the placental architecture [38], particularly the vascular characteristics, which are evident in women with diabetes.

Thrombosis of fetal vessels (Fig. 1-I)

This characteristic was identified when a thrombus partially or totally blocked a sizable fetal stem villous vessel [39]. In GDM, the blood arteries in a few terminal villi displayed occulting by thrombus [19;37].

Nucleated fetal RBCs (Fig. 1-J)

Erythropoietin levels rise because of tissue hypoxia, which in turn stimulates erythropoiesis and increases the quantity of nucleated fetal red blood cells in circulation.

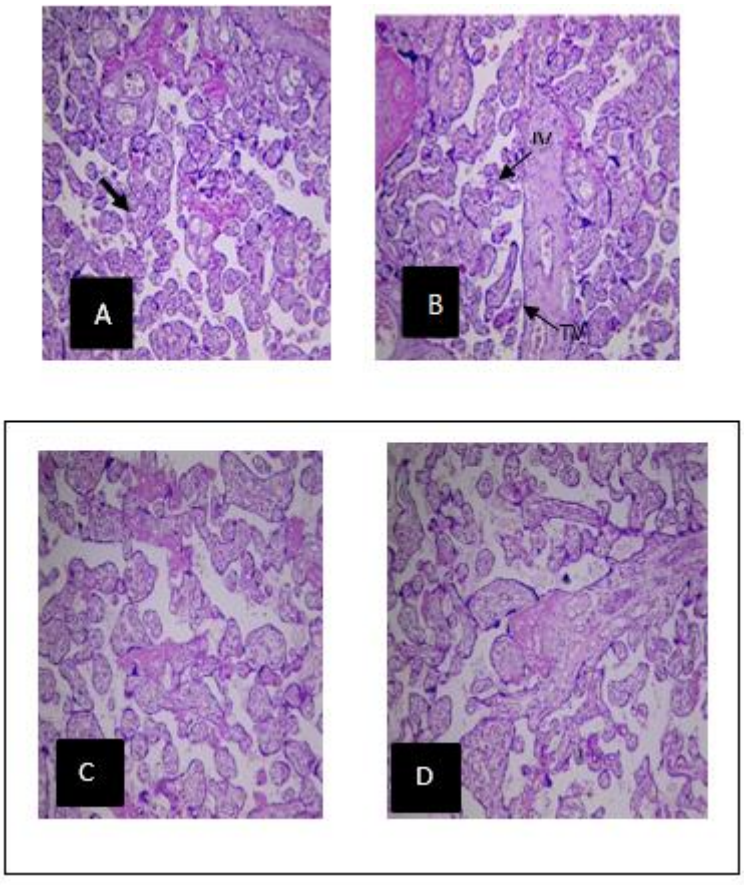
Increased erythropoiesis may result from both an immediate hemopoietic effect of hyperinsulinemia and an increase in erythropoietin levels [40].

Mature intermediate villi with a continuous layer of trophoblasts (Fig. 1-J):

In women with diabetes mellitus, this histological characteristic is proof of villous immaturity [26].

Villous edema (Fig. J)

fluid buildup in the placental villi's stroma. It was determined that the presence of aberrant deposits of mucopolysaccharides in the stroma of villi can result in the appearance of real villous edema in placentae of diabetic pregnancies because hyaluronic acid molecules have the unique ability to hold water [41].



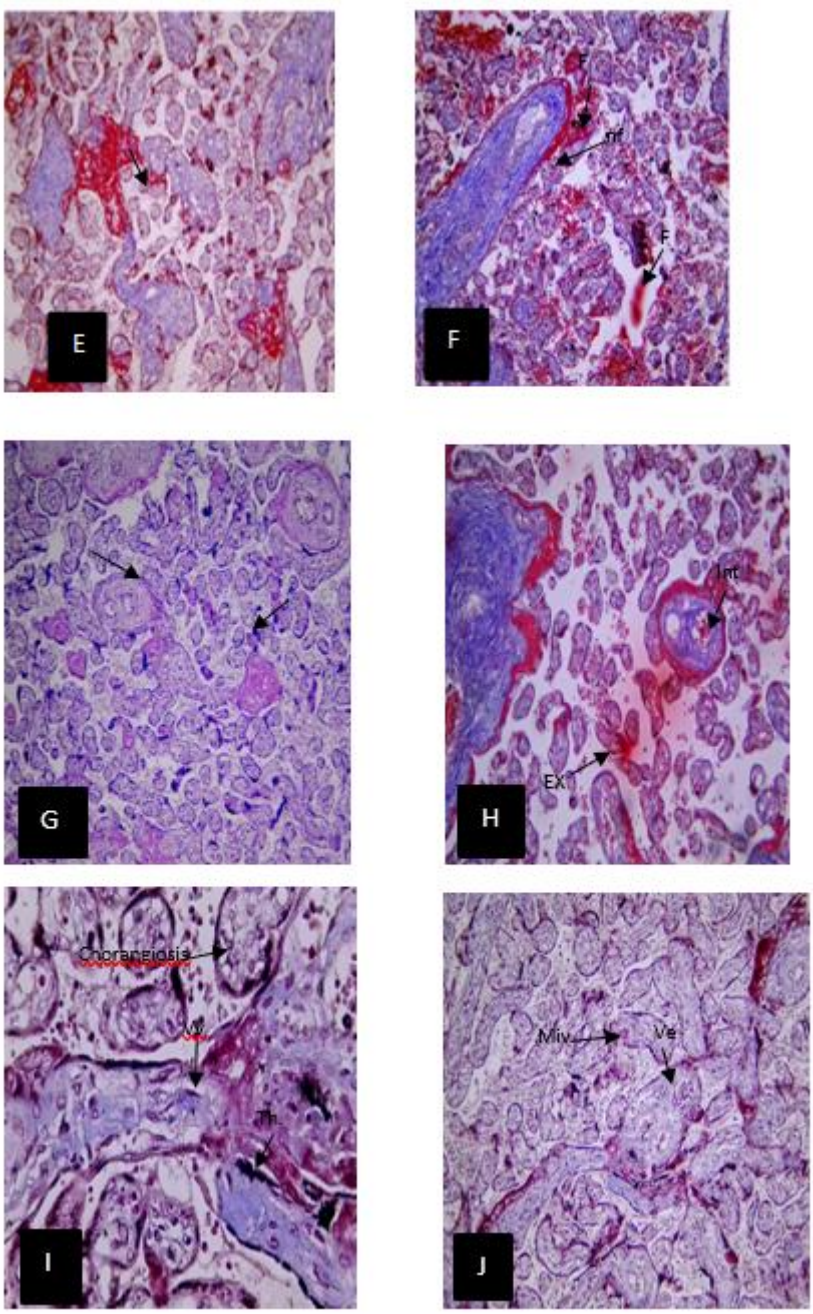


Figure 2:Histopathological findings in GDM placent: A-J Section of placenta in women complicated with DM showing: (A) Villi crowding. (B) An increase in the number of immature intermediate villi, Reduced density of terminal villi, Terminal villi size increase. (C) focal syncytial knots. (D) Terminal villi's cytotrophoblast, Reduced thickness of the vasculo-syncytial membrane. (E) Between the terminal villi, an extravillous fibrinoid. (F) Fibrinoid in the stem and terminal villi. (G) Immature intermediate villi with a higher density of Hofbauer cells and a looser reticular stroma. (H) Both external and intracellular calcification. (I) Chorangiosis, Villi vessel thickening, Thrombosis of fetal vessels (J) Mature intermediate villi with a continuous layer of trophoblasts, Villous edema.

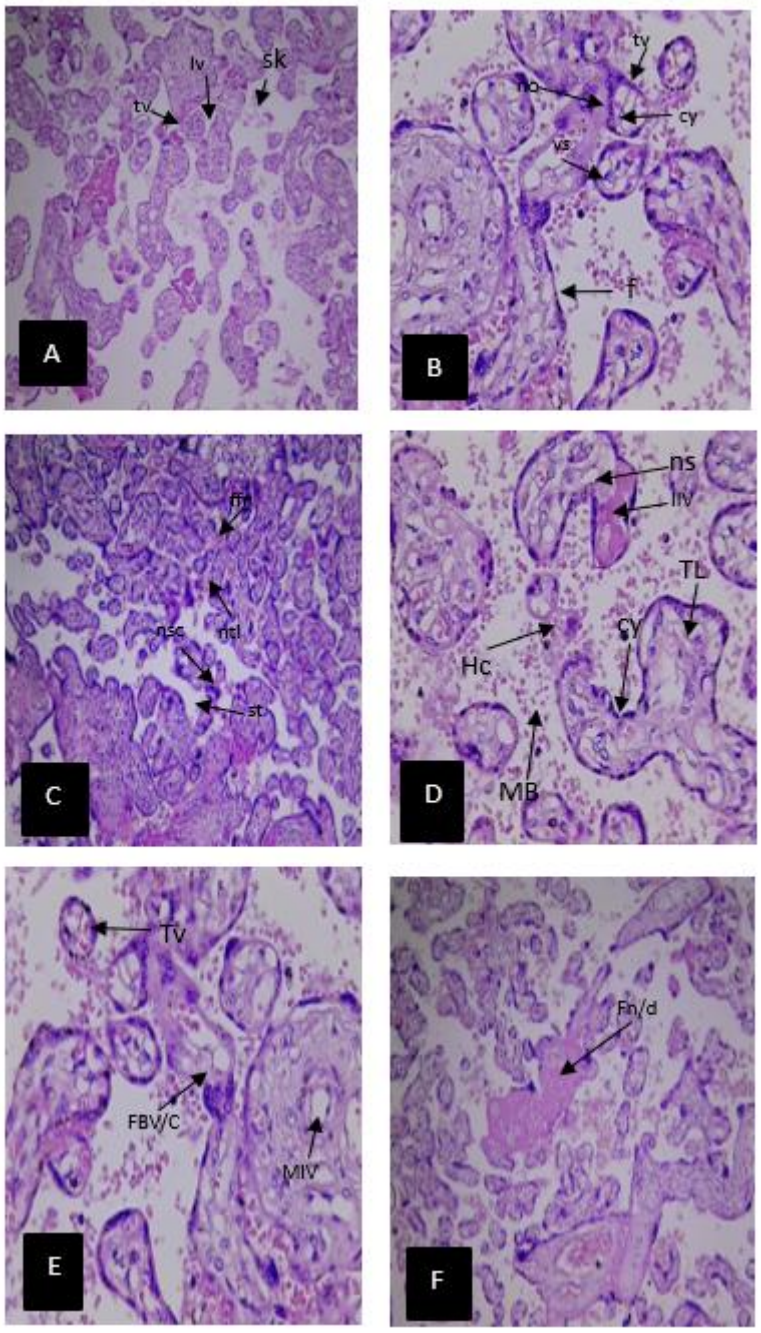


Figure 2: Histopathological findings in control placenta: The placenta's section in a normal woman shows: A) Normal immature intermediate villi, normal terminal villi density and size, normal crowded villi and small number of syncytial knots (10X). B) Terminal villi have a normal basement membrane, few cytotrophoblasts, normal vascular-syncytial membrane thickness, no extravillous fibrinoid between chorionic villi and fibrosis inside terminal and stem villi (40x). (C) little fibrinoid necrosis in the stem and terminal villi, No continuous trophoblastic layer, no indentation edge on the stem villi, and a normal respectable cell population in the stroma. (10x). (D) In immature intermediate villi, a normal syncytiotrophoblast, Hofbauer cells, Cytotrophoblast, Maternal blood in intervillous space, Trophoblastic layer(40x). (E) Mature intermediate villi, Terminal villi, Fetal blood vessels/capillaries(40x). (F) Fibrinoid necrosis/deposition(10X).

This study showed that VEGF expression was higher in placentas with gestational diabetes than in the non-diabetic group and that higher VEGF levels were associated with more severe instances of GDM. Patients with GDM showed comparatively high

expression of VEGF serological values and gene polymorphisms, according to a recent study that examined two groups of patients with GDM and those in good health based on serological numerical data [42]. It can be concluded that VEGF has a substantial connection with GDM based on the increased serological levels. The amount of VEGF that is expressed differently in the placenta and trophoblasts are examples of genetic orientations that are also important.

VEGF and placenta cells exhibited very varied activity, according to immunohistochemical methods. Syncytiotrophoblasts, stromal cells, and endothelial cells of placental arteries in GDM cases and the normal group are the cell types whose VEGF expression is analyzed. Normal women's placentas exhibit lower levels of VEGF positive. Despite the low density and small number of cells, syncytiotrophoblasts showed the highest activity in this group. Poor expressions were also seen in stromal placental cells. Positive expression was seen in a few positive cases (Table 1 and Figure 3). Bhattacharjee et al. found that in GDM placentas, VEGF expression was present mainly in syncytiotrophoblast, with moderate cytoplasmic staining, while other components (cytotrophoblast, stroma, vessels) showed little or no VEGF. In their series, VEGF immunoexpression in GDM placentas was "absent in all placental components except syncytiotrophoblast," indicating a trophoblast-dominant pattern. Other comparative IHC studies (e.g., Bolatay et al., and those summarized in reviews) similarly note increased VEGF and/or VEGFR-1 staining in trophoblastic cells in GDM and mild hyperglycemia, suggesting that syncytiotrophoblast responds directly to the hyperglycemic milieu by upregulating angiogenic signals [43].

In the GDM patients where it was expressed, VEGF immunohistochemistry was also authorized. It was strongly expressed in syncytiotrophoblasts usually (26, or 43%); it was also strongly expressed in endothelial cells (16, or 26%) and stromal cells (16, or 26%) (Table 1 and Figures 3). The VEGF expression in GDM cases changed when compared to the normal group, according to statistical analysis. Strong or moderate VEGF expressions in syncytiotrophoblasts are linked to an increased risk of GDM, whereas no or weak VEGF expression was seen in the normal group. VEGF immunoreactivity in stromal cells was significantly higher in the GDM group than in the control group. In GDM cases, endothelial cells in vascular arteries expressed themselves similarly.

Vascular Endothelial Growth Factor Expression in Iraqi Women's Placentas GDM-related complications

Table 1: VEGF expression in placenta of GDM cases and normal group by using microscopic analysis.

VEGF reactive	**normal group N =60 n(%)**	**GDM group N =60 n(%)**	**p-value between two groups**
Syncytiotrophoblast			
Non-expression	54(90%)	0	p=0.000**
Low	6 (10%)	0	p=0.0274*
Moderate	0	34(56.66%)	p=0.000**
Strong	0	26(43.33%)	p=0.000**
Stromal cells			
Non-expression	46(76.66%)	0	p=0.000**
Low	14(23.33%)	12(20%)	p=0.825***
Moderate	0	32(53.33%)	p=0.000**
Strong	0	16(26.66%)	p=0.000**
Endothelial cells			

Non-expression	42(70%)	0	p=0.000**
Low	18(30%)	10(16.66%)	p=0.130***
Moderate	0	34 (56.66%)	p=0.000**
Strong	0	16 (26.66%)	p=0.000**

***Statistically significant at the $p < 0.05$ level.**

****statistically highly significant. At p< 0.001.**

***** not statistically significant at $p < 0.05$.**

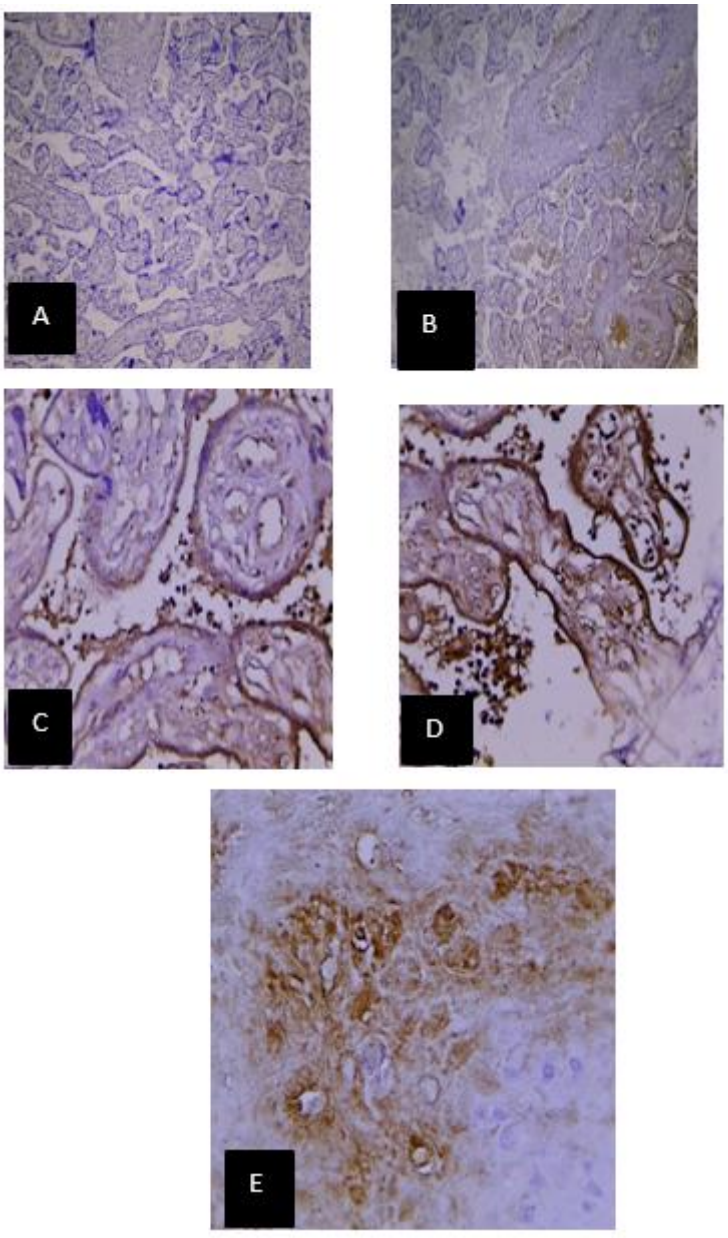


Figure 3: VEGF immunohistochemical expression: A-E: Section of placenta in women complicated with DM showing: (A) Section in the GDM placenta with immunohistochemical of VEGF showing no-expression Syncytiotrophoblast (S), stromal

cell (Sc), and endothelial cell (Ec), IHC, (10x). (B) showing weak expression Syncytiotrophoblast (S), stromal cell (Sc), and endothelial cell (Ec), IHC, (10x). (C) showing moderate expression in Syncytiotrophoblast (S), stromal cell (Sc), and endothelial cell (Ec), IHC, (40x). (D) showing strong expression in syncytiotrophoblast (S), and endothelial cell (Ec), IHC, (40x). (E) showing strong expression in stromal cell (Sc), IHC (40x).

Study of CD56 immunohistochemistry: No CD56 immunoreactivity was detected in any of the placental sections tested by the normoglycemic control group and the gestational diabetes mellitus (GDM) group. The examined tissue slices showed no evidence of membrane or cytoplasmic brown staining. The validity of the immunohistochemistry process was confirmed when positive-control tissues displayed the anticipated staining while negative controls displayed no specific signal. No comparison statistical analysis was carried out because every instance in both groups had an immunoreactivity score of 0 (table 2, figure 4).

Table 1: CD56 expression in placenta of GDM cases and normal group.

Variable	GDM Group, n/60 (%)	Control Group, n/60(%)	Statistical Test / p-value
CD56-positive cases	0/N (0%)	0/N (0%)	Not applicable
CD56 immunoreactivity score, median (range)	0 (0–0)	0 (0–0)	Not applicable

There was no statistical comparison between the GDM and control groups since all evaluable cases had a CD56 immunoreactivity score of 0.

Neither the GDM nor the normoglycemic control groups placental sections showed any CD56 immunoreactivity in this investigation. This result was regarded as technically legitimate since the positive and negative controls generated the anticipated staining patterns. Consequently, there was no discernible difference in CD56-positive cell infiltration between GDM and control placentas within the tissue compartments investigated in this investigation.

Decidual natural killer cells (DNK), which play significant roles in trophoblast invasion, spiral-artery remodeling, angiogenesis, and immunological control at the mother-fetal interface, are frequently identified by CD56. Although their quantity is highest in the early stages of pregnancy and significantly decreases as gestation progresses, some CD56-positive cells may continue to exist in the decidua of the third trimester [44,45].

The use of term placental samples, where DNK-cell density is physiologically decreased, may therefore be connected to the studys consistently negative CD56 results. The anatomical sampling site may also be reflected in the outcome. mother decidua, especially the decidua basalis and placental bed, contain most CD56-positive DNK cells; standard placental blocks may include little or no mother decidua. Therefore, the full absence of DNK cells from the maternal–fetal interface should not be inferred from the lack of CD56 staining in villous tissue [44].

GDM has been linked to altered NK-cell profiles, including modifications in CD56-positive NK-cell subsets, according to earlier research. Nevertheless, several biological specimens and techniques, including decidual tissue, placental extravillous areas, blood samples, flow cytometry, and multi-marker phenotyping, have been employed in these investigations. In placental regions from GDM pregnancies, for instance, Hara et al. observed greater proportions of CD16−CD56+ cells; however, newer research has found changed decidual NK-cell subgroups rather than universal changes visible by single-marker IHC. The disparity from the current findings may therefore be explained by variations in gestational age, sampling compartment, antibody technique, and definition of NK-cell subsets [46,47].

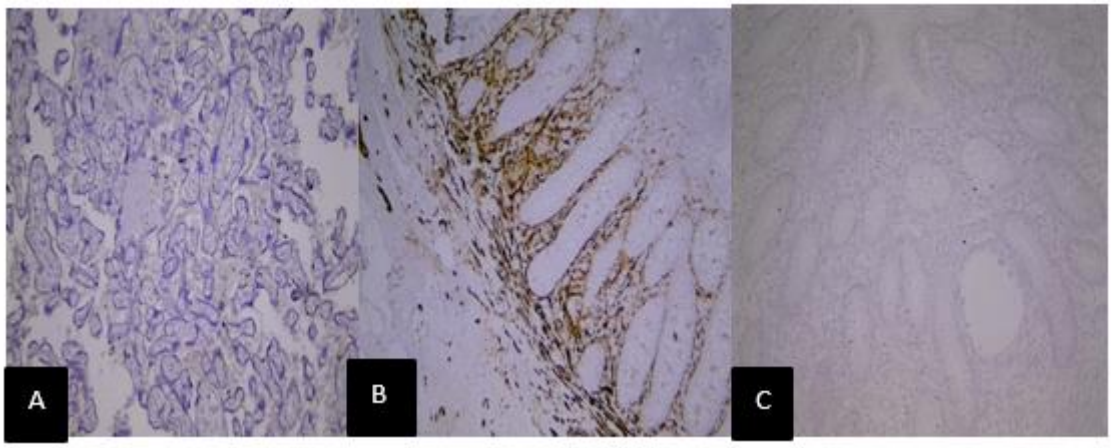


Figure 4: CD56 immunohistochemical staining in placental tissue. (A) Representative section from a GDM placenta showing absence of specific CD56 immunoreactivity. (B) Positive control showing the expected specific brown membranous/cytoplasmic staining. (C) Negative control shows absence of specific immunostaining. Hematoxylin counterstain; original magnification (10x).

Ethical Clearance: The Research Ethical Committee at scientific research by ministry of higher

education and scientific research

Conflict of Interest: The authors declare that they have no conflict of interest.

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the aim of the present study is to investigate the histopathological alternations in placentas from pregnancies complicated by gestational diabetes compared with normoglycemic pregnancies and to evaluate the immunohistochemical expression of vascular endothelial growth factor and CD56 in placental tissue from Iraqi women.

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