**ORIGINAL RESEARCH ARTICLE**

# Angiogenic imbalance, inflammation, and oxidative stress in patients with fetal growth restriction: A case-control study

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## Abstract

Fetal growth restriction (FGR) is a critical condition linked to various etiologies, increasing the risk of fetal complications and mortality. The study investigated the expression levels of angiogenic factors and inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) from normal pregnancies and those affected by FGR. It also examined their association with maternal oxidative stress markers. messenger RNA (mRNA) expression was analyzed using real-time PCR in PBMCs, while oxidative stress (OS) markers were assessed via spectrophotometric assays and ELISA in the maternal blood of 75 cases of FGR and 75 healthy pregnancies. FGR cases exhibited disrupted angiogenic signaling, elevated oxidative and inflammatory responses, indicating a pathological interplay among these systems. Results showed significantly reduced vascular endothelial growth factor (VEGF) along with increased soluble FMS-like tyrosine kinase-1 (sFlt-1) and nuclear factor-kappa B (NF-κB) expression in FGR cases. A notable inverse relationship of sFlt-1 existed with both VEGF and placental growth factor (PlGF), while sFlt-1 positively correlated with OS markers like Malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG). Additionally, NF-κB expression was strongly linked to MDA, tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6). This understanding highlights the potential for targeted interventions, such as antioxidant therapies or strategies to modulate inflammatory pathways, to improve outcomes in FGR. *(Afr J Reprod Health 2025; 29 [9]: 39-53).*

**Keywords:** Fetal growth restriction, Placental growth factor, Nuclear factor kappa B, Interleukin 6, Tumor necrosis factor alpha, Glutathione

## Résumé

Le retard de croissance fœtale (RCF) est une pathologie grave liée à diverses étiologies, augmentant le risque de complications et de mortalité fœtales. L'étude a examiné les niveaux d'expression des facteurs angiogéniques et des cytokines inflammatoires dans les cellules mononucléaires du sang périphérique (CMSP) issues de grossesses normales et de grossesses atteintes de RCF. Elle a également examiné leur association avec les marqueurs du stress oxydatif maternel. L'expression de l'ARN messager (ARNm) a été analysée par PCR en temps réel dans les CMSP, tandis que les marqueurs du stress oxydatif (SO) ont été évalués par spectrophotométrie et ELISA dans le sang maternel de 75 cas de RCF et de 75 grossesses saines. Les cas de RCF présentaient une perturbation de la signalisation angiogénique et des réponses oxydatives et inflammatoires élevées, indiquant une interaction pathologique entre ces systèmes. Les résultats ont montré une réduction significative du facteur de croissance de l'endothélium vasculaire (VEGF) ainsi qu'une augmentation de l'expression de la tyrosine kinase-1 soluble de type FMS (sFlt-1) et du facteur nucléaire kappa B (NF-κB) dans les cas de RCIU. Une relation inverse notable entre le sFlt-1 existait à la fois avec le VEGF et le facteur de croissance placentaire (PlGF), tandis que le sFlt-1 était positivement corrélé à des marqueurs de survie globale comme le malondialdéhyde (MDA) et la 8-hydroxy-2'-désoxyguanosine (8-OHdG). De plus, l'expression de NF-κB était fortement liée au MDA, au facteur de nécrose tumorale alpha (TNF-α) et à l'interleukine-6 (IL-6). Cette compréhension souligne le potentiel d'interventions ciblées, telles que les thérapies antioxydantes ou les stratégies de modulation des voies inflammatoires, pour améliorer les résultats dans le RCIU. *(Afr J Reprod Health 2025; 29 [9]: 39-53).*

**Mots-clés**: : Retard de croissance fœtale, Facteur de croissance placentaire, Facteur nucléaire kappa B, Interleukine 6, Facteur de nécrose tumorale alpha, Glutathion

## Introduction

The most commonly used characterization of fetal growth is fetal weight assessed by prenatal ultrasound screening.[1] About 40% of infants with fetal sizes below the 10th percentile, the threshold commonly used to diagnose fetal growth restriction (FGR), are fundamentally small and healthy. Contrarily, FGR is a pathology where the fetus is deprived of nutrients as well as oxygen.[2,3] FGR is found to affect about 3% to 7% of all pregnancies worldwide.[4] This not only increases the risk of fetal morbidity and mortality, [5,6,7] but also predisposes affected infants to a range of lifelong health issues, including hypertension, obesity, diabetes, and neurocognitive impairment.[8,9,10] These complications elevate cardiovascular risk and contribute to a major public health burden.[11,12] FGR can be linked to several maternal and fetal variables, including age, malnutrition, mother's height, smoking, obesity, systemic illnesses such as hypertension, genetics, and fetal anomalies.[13,14]

A key driver of FGR is placental insufficiency, often due to inadequate uterine spiral artery remodeling and poor placental development.[15,16] Normal placental development relies on numerous factors that stimulate and control villous angiogenesis and vascular maturation, including soluble FMS-like tyrosine kinase-1 (sFlt-1), vascular endothelial growth factor (VEGF), and placental growth factor (PlGF), which are vital for a highly bifurcated vascular meshwork.[17,18] While PlGF and VEGF are crucial proangiogenic growth factors, augmented sFlt-1 secretion, which self-attaches to both, reduces their availability.[19] It thus contributes to pregnancy complications due to defective placentation and uncontrolled free radical generation.[20,21,22,23] The resulting hypoxia and oxidative stress heighten nuclear factor-kappa B (NF-κB) expression, leading to increased inflammation and cytokine secretion such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) into the maternal circulation. [24,25,26] While the precise mechanisms causing angiogenesis imbalance and vascular remodeling in the uteroplacental unit are still being explored, the critical role of the VEGF family and inflammation in placentation abnormalities is well-established.[27]

Despite ultrasound being the current gold standard for diagnosing FGR, detection rates remain suboptimal. One study identified FGR in only 3.3% of cases, with suboptimal screening contributing to 80% of undiagnosed FGR cases, even after the 2013 French Guidelines were implemented. [28] While contemporary monitoring methods include recurrent fetal Doppler ultrasound and monitoring of heart rate, approximately 50% of FGR cases remain undetected during intrauterine stages, and conventional surveillance inadequately mitigates a substantial proportion of stillbirth occurrences. One of the main issues is the inaccurate measurement of symphysis-fundal height and the subjective misinterpretation of estimated fetal weight centiles.[29] Once the growth restriction sets in, options to prevent adverse outcomes become limited.[30] These challenges and deficiencies in current diagnostic and monitoring methodologies have prompted the imperative search for non-invasive biomarkers.[29] Measuring oxidative stress markers provides valuable insights into oxidative damage, lipid peroxidation, and antioxidant capacity, serving as crucial indicators for understanding disease mechanisms, assessing severity, and evaluating potential therapeutic interventions. Their analysis helps elucidate the link between oxidative stress, inflammation, and impaired fetal development, contributing to improved diagnostic and management strategies.

In this context, peripheral blood mononuclear cells (PBMCs) offer a minimally invasive, accessible, and ethically feasible alternative to placental tissue for studying molecular changes during pregnancy. PBMCs comprise lymphocytes and monocytes, key players in systemic immune and inflammatory responses, reflecting circulating signals and systemic alterations associated with placental dysfunction. In the past, studies have extensively assessed the mRNA expression of several placental tissue genes.[31,32,33,34,35] Gene expression in PBMCs has also been studied in pre-eclampsia[36,37,38,39,40]; however, only a few studies have specifically investigated FGR using PBMCs.[41]

Aiming to gain insight into the principal mechanisms of FGR with this non-invasive technique and create efficient therapies to enhance fetal outcomes, it was hypothesized that FGR cases would exhibit downregulation of proangiogenic genes and upregulation of antiangiogenic and pro-inflammatory genes in PBMCs, alongside elevated

maternal blood stress markers. These anticipated alterations would mirror known placental abnormalities in FGR and support the use of PBMCs as a non-invasive surrogate for studying placental dysfunction.

# Methods

## *Study population*

The case-control research study was conducted in line with the Declaration of Helsinki after receiving approval from the University of Lahore's Ethical Review Committee (Ref: CRiMIW2 2/Research/15). Sample size calculation was done using the following formula, with a power of 90%, a confidence interval of 95%, and a 5% incidence of IUGR.[42]

Where:

$$n = \frac{Z^2 P(1-P)}{(d)^2}$$

$$n = \frac{1.96^2 \; X \; 0.05(1-0.05)}{(0.05)^2}$$

Z = 1.96 (for 95% confidence), P = 0.05 (prevalence), d = 0.05 (margin of error)

The calculated sample size was 72.99; thus, 75 participants were included in each group to enhance statistical power.

The samples were obtained from tertiary care hospitals in Lahore from November 2021 to January 2023 after obtaining written consent from the participants. The cases included 75 pregnant women in their third trimester (31-40 weeks), diagnosed with FGR and 18 - 40 years of age; the controls were 1:1 age-matched and were normal pregnant women in the third trimester.

## *Inclusion criteria*

The study included pregnant patients and controls after the 30th week due to the broad aberrant indices relating to extreme prematurity before the third trimester. The study group had women between 31 and 40 weeks, where gestational age calculation was done by the date of onset of the last menstruation period and ultrasonographic findings. FGR was defined as an estimated fetal weight (EFW) under the 10th percentile for the given gestational age, based on INTERGROWTH-21st fetal growth standards, in conjunction with umbilical artery pulsatility index (PI) more than the 95th percentile on Doppler ultrasonography. These dual criteria helped distinguish true FGR in contrast to normally small fetuses.

## *Exclusion criteria*

Women with a history of cigarette smoking, kidney diseases, diabetes, arthritic conditions, a history of inflammatory bowel disease, cardiovascular conditions, and chronic conditions with inflammation were excluded. To control for confounding, all participants underwent screening for pregnancy-induced hypertension (PIH) and preeclampsia (PE). Blood pressure was measured using a calibrated sphygmomanometer at rest. A diagnosis of PIH was excluded if systolic blood pressure recorded <140 mmHg and diastolic <90 mmHg on at least two separate instances, and there was no proteinuria or other systemic signs. Patients with PE or chronic hypertension were excluded from both groups. Any pregnancies with genetic birth defects and fetal anomalies, as well as twin pregnancies, were also excluded for both the IUGR and healthy controls.

## *Participant enrollment and selection*

A total of 300 subjects were accessed for eligibility. Before the start of the study, an informed consent taken where the purpose and procedure were also explained. Two hundred and sixty participants consented to participate initially. However, one hundred and six participants were disqualified because they met the exclusion criteria and another four refused participation later on. In total, 75 controls and 75 IUGR patients were allocated to the respective case and control groups and were included in the analysis.

## *Sample collection*

Maternal venous blood samples (10 mL total) collection was done from all participants during antenatal checkups, specifically between 31-40 weeks of gestation. To minimize circadian and dietary variations in oxidative and inflammatory markers, all samples were drawn under fasting conditions in the morning hours (between 8:00 and 10:00 AM). Of the total volume, 5 mL was collected in a non-coated vacutainer (yellow top) for serum

separation, and the remaining 5 mL was collected in an EDTA-coated vacutainer for other analyses (e.g., PBMC isolation). Demographic data and complete medical, family, and obstetric histories were also meticulously recorded for each participant.

### *Oxidative stress markers*

**Malondialdehyde** (MDA) was measured by following the thiobarbituric acid reactive substances (TBARS) assay. Serum (94 µL) was aliquoted and optionally pre-incubated (37 °C) for 24 hours with 6 µL stock solution of copper chloride (or 6 µL acetic acid for controls) to induce oxidation. The oxidized samples (100 µL) were then mixed sequentially with 8.1% SDS (200 µL), sodium acetate buffer (1.5 mL), thiobarbituric acid (1.5 mL), and deionized water (700 µL) for a 4 mL final volume. After capping, incubated at 95 °C for one hour, later on ice for about thirty minutes, and then centrifuged (1500 x g for 10 minutes). Absorbance of the 150 µL supernatant was immediately noted at 532 nm, and concentrations were derived from a 1,1,3,3-tetramethoxypropane standard curve.[43]

**Glutathione** (GSH) levels were measured following a modified Ellman's method[44] as described by Stefanov *et al*. 2020.[45] Key modification to the original Ellman's method included initial sample (200 µL) deproteinization with 5% trichloroacetic acid and centrifugation at 12,000 x g for 10 minutes. The supernatant (0.1 mL) was mixed with pH 8.4 phosphate buffer (2 mL) and 5,5'-dithiobis-2-nitrobenzoic acid (0.5 mL DTNB) reagent along with distilled water (0.4 mL), vortexed, and later incubated for fifteen minutes at room temperature. Absorbance was recorded at 412 nm within 15 minutes. GSH levels were calculated from a standard curve and expressed in µmol/L.

**Glutathione Peroxidase** (GPx) was analyzed spectrophotometrically by monitoring the decrease of GSH using DTNB, as catalyzed by GPx in the presence of hydrogen peroxide ($H_2O_2$)[46]. Reaction medium with Tris-HCl buffer, EDTA, sodium azide, GSH, and $H_2O_2$ was prepared. Serum (50 µL) was added to the mix and incubated for 5 minutes at 37 °C. After incubation, the decrease in GSH was quantified by adding DTNB and measuring absorbance at 412 nm. Absorbance noted at 412 nm, and result stated as Units/L (after appropriate volume correction).

**Glutathione reductase** (GR) activity was measured spectrophotometrically, based on the use of NADPH as a coenzyme, which corresponds to a decline in absorbance (340 nm).[47] Glutathione reductase activity is expressed as the enzyme quantity required to reduce 1 micromole of oxidized glutathione (GSSG) per minute under standard conditions. Serum samples were thawed, mixed, and diluted subsequently in potassium phosphate buffer to achieve a concentration between 5-50 U/L. GR activity was measured in a mixture (1 mL) containing diluted serum (200 µL), GSSG solution (400 µL prepared in the same buffer), and freshly reconstituted NADPH (400 µL). For sample blanks, GSSG was replaced with buffer. Reactions were initiated by NADPH addition, mixed by pipetting, and absorbance was recorded at 340 nm after every minute for 5 minutes at 25 °C using a spectrophotometer. GR activity (U/L) was calculated from the net rate of NADPH consumption ($\Delta A_{340}$/min), using $\varepsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$ and a 5-fold dilution correction. All values fell within the assay's linear range (0.8-10.0 U/L).

**8-hydroxy-2'-deoxyguanosine** (8-OHdG) levels were measured using a competitive ELISA kit (Abcam ab285254, K4160). Serum was centrifuged and diluted 1:5 in the provided diluent. Diluted serum (50 µL) was added to the appropriate wells, and the Biotinylated Detection Antibody (50 µL) was added later. After incubation at 37°C for 45 minutes, wells were washed thrice. Subsequently, the working solution of HRP-Streptavidin Conjugate (SABC) was introduced and allowed to incubate at 37°C for 30 minutes. Following five washes, addition of TMB substrate (90 µL) was followed by incubation in the dark at 37°C for 15-30 minutes. The reaction was terminated with a stop solution (50 µL), measuring absorbance at 450 nm. Concentrations were derived from a standard curve.

### *mRNA expression of growth factors and inflammatory cytokines*

PBMCs were collected from EDTA-coated vacutainer blood samples within one hour of collection. Briefly, blood was diluted 1:1 (v/v) and PBMCs separated via Ficoll density gradient centrifugation. RNA extraction was done using the GeneJET RNA Purification Kit (Thermo Scientific, catalog no. K0731) following the instructions.

cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit. Gene expression was quantified by real-time PCR (qPCR) on a Bio-Rad CFX 96 system using cDNA and primers (supplementary tables 1 and 2).

The qPCR thermal cycling protocol included denaturation at 95°C for 30 seconds, 40 cycles at 94°C for 5 seconds and followed by annealing at 60°C for 30 seconds.

All the samples were assayed in duplicate with the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was selected as the reference gene for normalization due to its established stable expression in PBMCs across various physiological and pathological conditions, ensuring reliable relative quantification of target gene expression. [48] Relative gene expression was determined using the 2-ΔΔct method.

### *Statistical analysis*

Statistical analyses were conducted using IBM SPSS Statistics Version 22.0 (Armonk, NY, USA), and visualizations were generated using GraphPad Prism version 9.1 (San Diego, CA, USA). Gestational age at sampling ranged from 31 to 40 weeks and was collected during third-trimester prenatal visits.

Data distribution was checked using the Shapiro-Wilk test. The following mappings of statistical tests were applied based on data normality: Categorical and Ordinal Variables were assessed using the Pearson chi-square test or Fisher's exact test (if Cochran's assumptions were not met).

Continuous Variables (Normally Distributed) were analyzed using Student's independent t-test. Continuous Variables (Non-Normally Distributed) were assessed using the Wilcoxon-Mann-Whitney U test.

- Correlation Analysis: Pearson's correlation coefficient was used for relationships between normally distributed continuous variables. Spearman's rank correlation was considered for non-normal or ordinal variables. A p-value of $< 0.05$ was considered statistically significant for all comparisons.

## Results

### *Demographics*

The statistical summary of both groups' demographics, socioeconomic status, and obstetric history is below (Table 1). The FGR group showed a higher prevalence of individuals in extreme age groups. A significant association ($p<0.001$) was observed between BMI and FGR, with both underweight (9.3%) and obese (28%) women being more frequent in the FGR cohort compared to healthy pregnant women. Furthermore, a majority of FGR women (80%) reported a monthly household income below the minimum wage. At the time of sampling, a substantially higher proportion of FGR patients were preterm (92%) compared to the control group. While no overall association was found between gravidity and FGR, a higher frequency of multigravida women (85.3%) was noted within the FGR group. A significant difference ($p < 0.001$) was also observed in the previous history of abortions and intrauterine growth restriction (IUGR) in FGR females compared to healthy controls. No association was found between FGR and parity ($p = 0.167$). Despite the exclusion of chronic hypertension, pregnancy-induced hypertension (PIH), and preeclampsia, significant differences were observed in the mean systolic ($p < 0.001$) and diastolic ($p < 0.001$) blood pressure between FGR cases and controls.

### *Gene expression analysis*

In the FGR group, gene expression analysis demonstrated a significant upregulation of sFlt-1, NF-κB, and inflammatory cytokines TNF-α and IL-6, alongside a notable downregulation of proangiogenic factors PlGF and VEGF (Figure 1). The independent t-test was applied to compare mean fold change among the groups. The mean fold changes for sFlt-1 ($2.30 \pm 0.40$), TNF-α ($2.02 \pm 0.27$), IL-6 ($2.56 \pm 0.51$), and NF-κB ($1.92 \pm 0.34$) were significantly higher compared to controls ($p <0.001$),whereas PlGF ($0.66 \pm 0.16$) and VEGF($0.63 \pm 0.30$) were significantly decreased ($p < 0.001$).

**Table 1:** A comparison of demographics among controls and FGR

| Demographics | Control (n=75) | FGR (n=75) | p-value |
|---|---|---|---|
| **Maternal Age (years)** | 25.1± 3.84 | 27.96 ± 5.44 | < 0.001*** |
| **Gestational Age (weeks)** | | | |
| Preterm ≤ 36 | 39 (52.0%) | 69 (92.0%) | < 0.001*** |
| Term 37-38 | 23 (30.7%) | 6 (8.0%) | |
| Full term 39-40 | 13 (17.3%) | 0 (0.0%) | |
| **Gravidity** | | | |
| Primigravida | 15 (20.0%) | 11 (14.7%) | 0.518(NS) |
| Multigravida | 60 (80.0%) | 64 (85.3%) | |
| **Parity** | | | |
| Nullipara | 18 (24.0%) | 23 (30.7%) | 0.167 (NS) |
| Primipara | 27 (36.0%) | 17 (22.7%) | |
| Multipara | 30 (40.0%) | 33 (44.0%) | |
| Grand multipara | 0 (0.0%) | 2 (2.7%) | |
| **BMI kg/m$^2$** | | | |
| Underweight | 4 (5.3%) | 7 (9.3%) | < 0.001*** |
| Healthy | 41 (54.7%) | 26 (34.7%) | |
| Overweight | 29 (38.7%) | 21 (28.0%) | |
| Obese | 1 (1.3%) | 21 (28.0%) | |
| **Socioeconomic status** | | | |
| Low income | 49 (65.3%) | 60 (80.0%) | 0.044* |
| Moderate income | 26 (34.7%) | 15 (20.0%) | |
| **Abortion** | 0 (0-2) | 0 (0-9) | < 0.001*** |
| **Previous IUGR** | 0 (0-1) | 0 (0-2) | < 0.001*** |
| **Systolic Blood Pressure (mmHg)** | 116.51 ± 6.15 | 128.80 ± 9.46 | < 0.001*** |
| **Diastolic Blood Pressure(mmHg)** | 70.03 ± 3.78 | 79.36 ± 4.64 | < 0.001*** |

Note. Data is presented as proportions with corresponding percentages (%), median (range) or mean ± standard deviation. Normality was assessed using the Shapiro-Wilk test. For normally distributed continuous variables (maternal age, systolic and diastolic blood pressure), the student's t-test was used to compare means between groups. The Chi-square test was employed for the comparison of categorical variables between groups. Abbreviations BMI-body mass index (underweight <18.5, normal 18.50 - 24.99, overweight > 25, obese > 30).[49] **p-value <0.05, **p <0.01, ***p <0.001 was considered as significant*

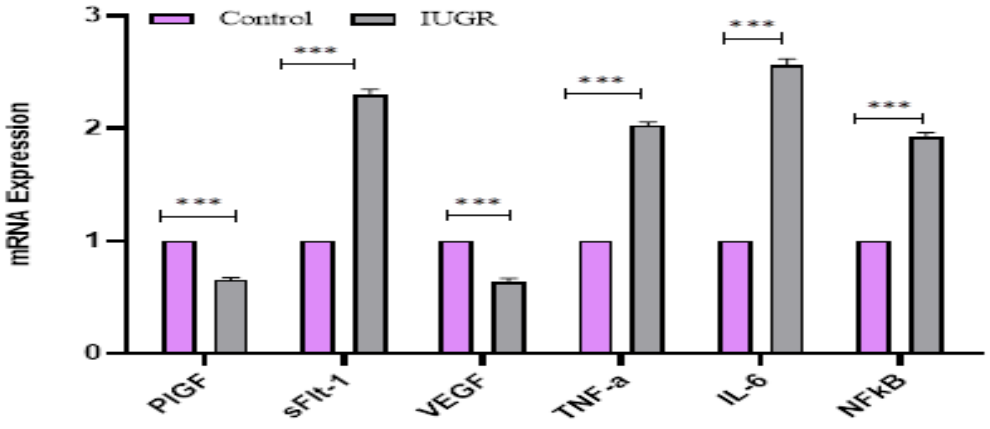


**Figure 1:** Relative mRNA expression (fold change on y-axis) was calculated using the ΔΔCt method, normalized to housekeeping genes, and expressed relative to the control group. Mean ± SEM (standard error of the mean). SEM is a statistical measure that estimates how far the sample mean of the data is likely to be from the true population mean.
The 2-ΔΔct method was used for relative gene expression analysis.
**p-value <0.05, **p <0.01, ***p <0.001 was considered as significant*

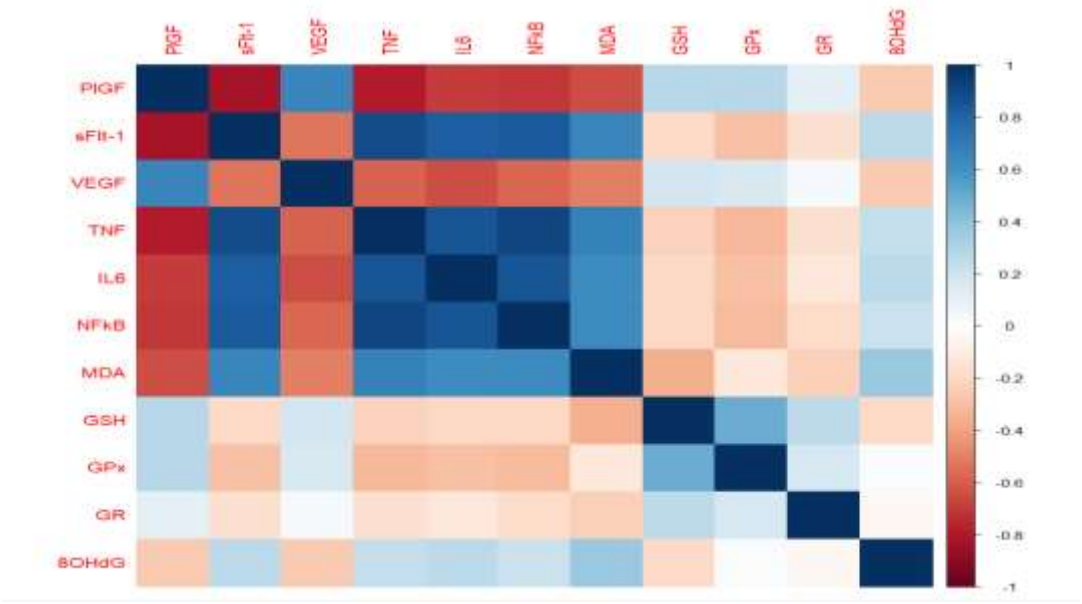


**Figure 2:** Heatmap showing the Pearson correlation matrix between mRNA expression levels of angiogenic factors (PlGF, sFlt-1, VEGF), inflammatory cytokines (TNF-α, IL-6, NFκB), and oxidative stress biomarkers (MDA, GSH, GPx, GR, 8-OHdG).
Color scale indicates strength and direction of correlation (r), ranging from +1 (strong positive, blue) to -1 (strong negative, red). A darker shade indicates a stronger correlation.
**p < 0.001, **p < 0.01, *p < 0.05 was considered as significant (two-tailed Pearson correlation test)*

**Table 2:** A comparison of serum oxidative stress marker levels between groups

| Variable | Group | Mean±SD | Minimum | Maximum | p-value |
|---|---|---|---|---|---|
| MDA | Control | 1.19 ± 0.52 | 0.11 | 2.41 | |
| (nmol/ml) | Cases | 2.82 ±0.90 | 0.98 | 4.46 | <0.001*** |
| GSH | Control | 8.39 ±1.88 | 4.16 | 12.70 | |
| (µmol/L) | Cases | 7.44 ± 1.73 | 1.76 | 10.68 | 0.002** |
| GPx | Control | 7.36 ± 1.24 | 4.89 | 9.97 | |
| (U/L) | Cases | 6.71±0.61 | 5.05 | 8.11 | <0.001*** |
| GR | Control | 2.61 ± 0.60 | 1.03 | 3.68 | 0.041* |
| (U/L) | Cases | 2.40 ± 0.67 | 1.13 | 3.99 | |
| 8-OHdG | Control | 40.14± 9.15 | 21.73 | 58.66 | <0.001*** |
| (ng/ml) | Cases | 45.68±8.09 | 22.11 | 65.01 | |

Note. Data is listed as mean ± standard deviation and range (minimum-maximum).
U/L refers to: The amount of enzyme (units) present in one liter of solution that catalyzes the conversion of one micromole of substrate per minute under standard conditions.
**p-value <0.05, **p <0.01, ***p <0.001 was considered as significant*.

Pearson correlation analysis revealed strong and statistically significant associations supporting the interplay between angiogenic imbalance and inflammation in FGR **(**Figure 2). Notably, sFlt-1 expression correlated negatively with PlGF (r = -0.83, p < 0.001) and VEGF (p < 0.001), and positively with TNF-α (r = 0.88), IL-6 (r = 0.83), and NF-κB (r = 0.84), all with p < 0.001.

Similarly, NF-κB expression correlated strongly with TNF-α (r = 0.91) and IL-6 (r = 0.86) (p < 0.001), consistent with coordinated inflammatory activation. TNF-α and IL-6 were also positively correlated (r = 0.86, p < 0.001). These findings

collectively suggest a robust link between elevated anti-angiogenic signaling and inflammatory pathways in FGR pathophysiology.

### *Redox imbalance*

The results revealed higher mean serum MDA and 8-OHdG ($p<0.001$) in the FGR women, showing increased lipid peroxidation as well as DNA damage (Table 2).

However, the decreased serum levels of GSH ($p =.002$), GSH-Px ($p<0.001$), and GR ($p =.041$) showed overall reduced antioxidant capacity in the patients. Pearson's correlation method analyzed the correlations among biomarkers (Figure 2). GPx is also moderately correlated to GSH ($r =.493$). MDA also shows a moderately positive correlation with 8-OHdg, $r =.375$. A moderate negative relation coexisted between MDA and GSH, $r (75) = -.358$, $p < 0.001$.

### *Correlations*

This investigation revealed compelling correlations among growth factors, stress markers, and inflammatory cytokines, highlighting intricate interconnections within placental function. Notably, MDA, a key marker of lipid peroxidation, showed strong inverse relationships with proangiogenic factors (PlGF and VEGF) and strong positive associations with sFlt-1 and inflammatory mediators (TNF-α, IL-6, and NF-κB). These findings highlight the link between heightened oxidative stress and impaired placental vascular function, likely contributing to FGR pathology. while displaying strong positive correlations with sFlt-1, TNF-α, NF-κB , and IL-6 according to Cohen's (1988) criteria[50]. The results indicate a robust correlation between lipid peroxidation and elevated sFlt-1 levels and a noteworthy association with inflammatory cytokines, influencing the NF-κB pathway. Antioxidant markers, particularly GPx and GSH, were inversely correlated with sFlt-1 and inflammatory cytokines, underscoring the insufficient antioxidant defense in affected pregnancies. Additionally, GSH was positively associated with PlGF, reinforcing its role in supporting placental angiogenesis. Further 8-OHdG, a marker of oxidative DNA damage, showed positive associations with sFlt-1 and inflammatory cytokines, and inverse associations with angiogenic factors. These findings support a model in which oxidative stress not only disrupts angiogenesis but also contributes to inflammation and cellular injury in the placental environment.

## Discussion

Maternal health and physiological factors profoundly influence placental development and angiogenesis, with their disruption driving serious pregnancy complications including miscarriage, stillbirth, FGR, PE, and preterm birth.[51,52,53] The study investigated the extraplacental growth factors, inflammatory cytokine expression in PBMCs of women suffering from FGR, and stress biomarkers. Alongside DNA, the mRNA derived from the placenta is found in the mother's bloodstream, providing a broader spectrum of opportunities for monitoring the function of the placenta.[54] Numerous RNA transcripts are unique to the placenta, and these circulating placental RNAs may exhibit distinct expression patterns in complicated pregnancies caused by early-onset preeclampsia (EOP) and FGR.[26,55] Particles from the syncytiotrophoblast can enter the maternal blood via apoptosis or necrosis on the surface of the placenta or through the formation of micro-vesicles. In normal pregnancies, exiled trophoblasts and their structures have been found in uterine blood, exhibiting transcriptional activity and contributing significantly to the synthesis of proteins and placental mRNA.[56,57] Deviations from normalcy (due to the presence of risk factors) in placentation and the invasion of spiral arteries can result in oscillatory disturbed shear, resulting in an elevation of circulating sFlt-1, a spliced form of VEGF receptor-1, deficient in both the cytoplasmic and the transmembrane domains. sFlt-1 acts as an antagonist to the angiogenic effects of both VEGF and PlGF by binding to them, thus disrupting normal angiogenesis.[58]

This investigation revealed a 2.3-fold increase in sFlt-1 mRNA expression alongside significantly reduced VEGF and PlGF expression in PBMCs from FGR cases. These findings resonate with Spiel *et al*.[59] who also noted increased sFlt-1 mRNA expression in twenty-eight percent of non-preeclamptic (small for gestational age) pregnancies, suggesting a shared pathophysiology rooted in angiogenic imbalance. Similarly, Gaccioli *et al*.[60] identified elevated placental sFlt-1 and reduced PlGF in the maternal serum of women with FGR,

underscoring disrupted angiogenesis. While Hoeller *et al*.[20] reported unchanged placental PlGF mRNA in FGR, their acknowledgment of increased sFlt-1 points to a potential mechanism where enhanced sFlt-1 binding contributes to reduced free circulating PlGF. The study, however, involved only seven IUGR cases, six cases of preeclampsia, and six age-matched controls (a relatively smaller sample size).

This binding mechanism is further supported by Wallner *et al*.[61] and Schlembach *et al*. [62] who proposed that reduced placental size and increased sFlt-1 production could account for diminished circulating PlGF. Recent research by Mitlid-Mork *et al*.,[63] who observed significantly lower maternal PlGF and higher VEGFR-1 (sFlt-1) levels in pregnancies complicated by placental syndromes, including FGR, further supports the current study's findings. In addition, Fuenzalida *et al*.[64] deduced that hypoxia-induced upregulation of sFlt-1 and PlGF imbalance, in association with oxidative stress in trophoblast cells, may underlie the pathogenesis of FGR. These observations reinforce the hypothesis that trophoblast hypoxia and placental dysfunction drive the dysregulation of angiogenic factors. The consistency between our PBMC-based gene expression data and placental studies highlights the potential of PBMCs as a noninvasive surrogate marker for placental dysfunction. Furthermore, these angiogenic imbalances, particularly sFlt-1 upregulation, may have diagnostic relevance for identifying pregnancies at heightened risk for FGR.

The study by Ravikumar *et al*.[27] is further in support by demonstrating significantly lower placental VEGF and PlGF transcript abundance in FGR placentae compared to appropriate for gestational age (AGA) placentae. While their work did not report significant differences in placental FLT1 mRNA, this difference highlights an important aspect: while placental mRNA levels of FLT1 might not show significant changes, the soluble form (sFlt-1), or its expression in PBMCs, might be distinctly elevated. The general reduction in pro-angiogenic factors (VEGF, PlGF) at the placental level aligns with this study's PBMC-based observations, reinforcing the concept of angiogenic imbalance in FGR. Interestingly, their finding of increased placental PlGF transcripts in hypoxic IUGR placentae suggests complex localized responses to stress, which complements our systemic PBMC findings. Similarly, Benton *et al*. [65] observed significantly low PlGF levels in all nine IUGR cases they examined, leading them to suggest PlGF as a potential antenatal marker for placental IUGR.

The role of sFlt-1 in promoting apoptosis, oxidative stress, and ER stress has been widely documented.[66] Several studies have reported elevated oxidative stress in FGR, marked by increased protein carbonyls and reduced antioxidant levels.[67] Elevated plasma MDA levels in IUGR patients have also been consistently observed.[68] Supporting the present study's findings, a study comparing 20 FGR cases and 20 healthy pregnancies found significantly higher MDA in serum but lower levels in myometrial tissue, indicating compartment-specific oxidative imbalance[69]. Aljaser *et al*. similarly reported elevated MDA and reduced antioxidant capacity in FGR, consistent with the present study.[70]

Conversely, Zygula *et al*. found no difference in plasma antioxidant enzyme levels but observed elevated total antioxidant capacity (ORAC) in plasma and saliva, and increased GPx levels in IUGR cases. Notably, their exclusion of hypertensive patients may account for the absence of differences in plasma oxidative stress markers. In contrast, our study also excluded individuals with hypertension, yet still revealed a significant difference in blood pressure between the two groups.[71] Celik et al. reported similar oxidative stress patterns in FGR and uncomplicated pregnancies, aligning partially with Zygula's observations.[72] In contrast, Singh *et al*.[73] found significantly higher 8-OHdG levels in IUGR cases compared to non-pregnant women, supporting our findings of oxidative DNA damage. Another research showed significantly elevated 8-OHdG levels in preeclampsia, particularly when accompanied by IUGR ($p = 0.006$), which further supports our results by reinforcing the role of oxidative DNA damage in the pathophysiology of FGR.[74]

The Nuclear Factor-Erythroid 2 p45-Related Factor (NRF2) regulates antioxidant defense by forming a complex with Kelch-like ECH-Associated Protein 1 (KEAP1), translocating to the nucleus, and activating antioxidant response element (ARE)-driven genes. These genes encode phase II detoxification enzymes and antioxidant proteins. Under oscillatory disturbed shear stress, as seen in

placental insufficiency, NRF2/ARE signaling is suppressed, leading to NF-κB activation and upregulation of inflammatory genes. When oxidative stress is moderate, KEAP1 oxidation is reversible, allowing NRF2 to restore redox balance. However, in severe stress, NRF2 may paradoxically increase ROS, irreversibly altering KEAP1 and activating NF-κB, thereby promoting proinflammatory cytokine expression.[75,76]

The current study's findings align with Kirici *et al*., who reported elevated NF-κB in FGR patients, although IL-6 levels did not differ significantly.[77] Al-Azemi *et al*. observed elevated TNF-α, IL-6, and IL-4 and reduced anti-inflammatory IL-10 in IUGR placentas, highlighting a proinflammatory shift.[78] Similarly, Kaya *et al*. found increased TNF-α, IL-6, MDA, and reduced antioxidant enzyme levels in FGR rat models.[79] In contrast, some studies found no significant differences in TNF-α and IL-6 between IUGR and normal pregnancies.[80] Zygula *et al*. also reported an absence of inflammatory markers in IUGR, possibly due to participant selection limited to normotensive cases. Prolonged oxidative and ER stress may occur in severe placental ischemia-reperfusion scenarios, exacerbated by preexisting conditions such as poor diet, nutritional deficiencies, or genetic polymorphisms. These factors may converge to drive both FGR and maternal hypertension.[81]

This is the first-ever study in Pakistan that measured the correlation of relative expression of the growth factors, inflammatory cytokines in the PBMCs and stress markers in IUGR patients as far as we are aware. To the best of our knowledge, the study is the first to report mRNA expression of angiogenesis and inflammation in PBMCs of FGR patients worldwide. PBMCs instead of placental tissue offer significant ethical and practical advantages, as they are more easily accessible via non-invasive methods, reducing risks to both mother and fetus-especially in the third trimester. PBMCs offer a non-invasive, practical alternative to placental tissue, especially in late pregnancy. Although they do not capture placental site-specific changes, PBMCs reflect systemic effects of placental dysfunction. Placenta-derived microvesicles and circulating RNA can modulate PBMC gene expression, allowing these cells to serve as peripheral markers of placental stress. This enables the detection of downstream alterations related to angiogenesis and inflammation in IUGR. This choice may limit insights into specific placental responses unique to IUGR. However, we acknowledge the importance of comparing differential expression across tissue, PBMCs, and serum to further validate our findings. The gestation time can significantly influence the study's findings. We have only selected the patients in the third trimester. Analyzing the results obtained before the 30th gestational week would be challenging due to the broader spectrum of abnormal indices within this timeframe associated with prematurity. Differences in exposure to various environmental factors (ionizing and UV radiations, heavy metals, pollutants) and physical activity can influence the results. Occupation and environment can significantly affect disease severity, such as the association with organochlorine pesticides.[82] The current study did not collect physical activity, environmental, or occupational stressors data. It also did not analyze the diet and protein intake of the patients, which can influence the disease status and severity.[83,84]

## Conclusions

In conclusion, the study highlights the significance of maternal factors in manifesting an antiangiogenic environment. The findings of this study shed light on the complex and interconnected mechanisms underlying FGR. Elevated expression of sFlt-1, coupled with raised stress markers (MDA, 8-OHdG) and reduced antioxidant capacity (low GSH, GPx and GR), contribute to an antiangiogenic environment. Notably, the variable endothelial dysfunction and inflammatory response observed in FGR appears to be linked to the degree of stress.

Importantly, the use of peripheral blood mononuclear cells (PBMCs) offers a non-invasive window into placental dysfunction, capturing systemic molecular changes associated with FGR. These findings support the potential clinical utility of circulating biomarkers such as sFlt-1, inflammatory cytokines, and oxidative stress indicators for early identification and risk stratification of FGR pregnancies.

Moreover, the observed correlation between the degree of oxidative stress and endothelial/inflammatory markers highlights possible avenues for targeted therapeutic interventions. Together, these insights enhance our

mechanistic understanding of FGR and pave the way for biomarker-driven diagnostics and management strategies aimed at improving perinatal outcomes.

## Authorship contribution

Conceptualization, Mehwish Iftikhar, Malik Ihsan Ullah Khan; Sumaira Sharif, Methodology, Mehwish Iftikhar, Sawar Khan; Software, Sawar Khan; Validation, Madeeha Shahzad Lodhi, Syed Imran Ali Shah; Formal analysis, Sawar Khan, Syed Imran Ali Shah; Investigation, Mehwish Iftikhar; Resources, Malik Ihsan Ullah Khan, and Sumaira Sharif; Data curation, Mehwish Iftikhar; Writing, original draft preparation, Mehwish Iftikhar; Writing, review and editing, Mehwish Iftikhar, Syed Imran Ali Shah; Visualization, Malik Ihsan Ullah Khan; Supervision, Malik Ihsan Ullah Khan and Sumaira Sharif; Funding acquisition, Malik Ihsan Ullah Khan and Sumaira Sharif. All authors have read and agreed to the published version of the manuscript.

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## Data availability

All data is presented in the paper, and raw data is available on request.

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## Ethics declarations

The Bioethical, Biosafety and Biosecurity Committee (BBBC) reviewed the research topic/ paper at every aspect concerning ethical, biosafety, and biosecurity issues at the institute and around. The committee decided on Dr. Mehwish Iftikhar (DBC 02173002) to conduct research work and submit her article for publication in an international journal. Ref: CriMM/22/15 / Dated: 18-04-2022.

## Competing interests

The authors declare that they have no conflicts of interest.

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**Supplementary Table 1:** The sequence of primers for genes involved in angiogenesis with housekeeping gene.

| Gene | Sequence | PCR Product Length (bp) |
|---|---|---|
| VEGFA-FP | 5'-TGC AGA TTA TGC GGA TCA AAC C- 3' | 81-bp |
| VEGFA-RP | 5'-TGC ATT CAC ATT TGT TGT GCT GTA G-3' | |
| sFlt-1-FP | 5'-GGC TGT TTT CTC TCG GAT CTC- 3' | 158-bp |
| sFlt-1-RP | 5'-CAT CTC CTC CGA GCC TGA AAG-3' | |
| PlGF-FP | 5'-ACG TGG AGC TGA CGT TCT CT-3' | 241-bp |
| PlGF-RP | 5'-CAG CAG GAG TCA CTG AAG AG-3' | |
| GAPDH-FP | 5'-ACG GAT TTG GTC GTA TTG GG-3' | 214-bp |
| GAPDH-RP | 5'-CGC TCC TGG AAG ATG GTG AT-3' | |

**Supplementary Table 2**: Sequence for primers for genes involved in inflammation.

| Gene | Sequence | PCR Product Length (bp) |
|---|---|---|
| TNFα-FP | 5'-GGA GAA GGG TGA CCG ACT CA-3' | 362-bp |
| TNFα-RP | 5'-CTG CCC AGA CTC GGC AA-3' | |
| NFκB-FP | 5'-TAC TCT GGC GCA GAA ATT AGG TC-3' | 264-bp |
| NFκB-RP | 5'-CTG TCT CGG AGC TCG TCT ATT TG-3' | |
| IL-6-FP | 5'- GGT ACA TCC TCG ACG GCA TCT-3' | 81-bp |
| IL-6-RP | 5'- GTG CCT CTT TGC TGC TTT CAC - 3' | |