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Study of the Vascular Endothelial Growth Factor Gene Expression and Serum Placental Growth Factor Levels in Egyptian Women with Preeclampsia

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Abstract:
Introduction: Preeclampsia (PE) is one of the major causes of feto-maternal morbidity and mortality, it is diagnosed by development of hypertension and proteinuria after 20 weeks of gestation. This study was conducted to determine whether altered levels of vascular endothelial growth factor (VEGF) andPLGF level may play a role in the pathogenesis of preeclampsia.**Methods**: We performed a cross section study in 50 preeclampticwomen: 25women with mild PE, 25 patients with severe PE. While 24 normotensive pregnant women matched for age as a control group. Placental mRNA expression of VEGF was measured by real time PCR (RTPCR).Maternal serum levels of PLGF was measured by ELISA.**Results:** Themean VFGF expression was statistically higher in cases of severe PE than Mild PE, both were higher than control group ($p<0.0005$). On the other hand the mean PLGF levels wasstatistically significantly higher in control group as compared to either of the two PE groups ($p< 0.0005$) and there is no statistically significantly difference between sPE and mPE groups. **Conclusion:** The increase of VEGF gene Expression in placenta tissue and decrease of PLGF level in maternal serum predict the risk of preeclampsia.

Keywords: VEGF, PLGF, gene expression, Preeclampsia.

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I. INTRODUCTION

Preeclampsia (PE), which is diagnosed by hypertension and proteinuria after 20 weeks of gestation, is one of the major causes of fetomaternal morbidity and mortality (1). The exact pathophysiology of PE remains still unknown, but is closely related to development of placenta (2). Abnormal placental development due to disturbance in angiogenesis and impaired trophoblast invasion leads to perfusion disorder in the uteroplacental compartment with resultant hypoxia and endothelialdysfunction. PLGF is a proangiogenic protein with an important role in the process of angiogenesis and embryogenesis, it binds to vascular endothelial growth factorreceptor 1 and displaces VEGF to bind to vascular endothelial growth factor receptor 2 (VEGFR-2), the major receptor of signaling cascades in angiogenesis, expressed in cytotrophoblast(3). VEGFA and PIGF actingthrough their receptors VEGFR1 (FLT1—Fms-like tyrosinekinase receptor-1), VEGFR2 (Kinase insert domainreceptor—KDR) are considered most important in regulating early placental vascular development during pregnancy (4).Vasculogenesis and angiogenesis are considered to be central processes in the development of the placenta and are mainly controlled by vascular endothelial growth factor (VEGF). Altered levels of VEGF and its receptors can disrupt angiogenesis, leading to placental insufficiency and endothelial dysfunction observed in PE (5). SerumVEGF levels in the maternal circulation were elevated in women with PE versus uncomplicated pregnancies (6). However, there is substantial discrepancy in the literature concerning levels of placental VEGF in PE pregnancies (7, 8).

Placental VEGF mRNA levels have been reported to be decreased, increased or unchanged in PE cases versus uncomplicated controls. So, we undertook this study to determine the level of PLGF in maternal serum and expression of VEGFA mRNA in placentae of PE in order to clarify the various discrepancies and gain further evidence for its role in Preeclampsia.

II. PATIENTS AND METHODS:

Blood Collection and Patients

Fifty patients diagnosed with Preeclampsia were collected from theObstetrics and Gynecology department, Mansoura University, Egypt (Their mean age (years) ± SD were 28.15 ± 5.25) during the period from July 2015 and May 2017.Preeclamptic women were divided into 25 women diagnosed as mild preeclampsia (MPE) and 25 women were diagnosed as severe preeclampsia (SPE). The control group comprised 24 normotensive pregnant women The blood samples were taken from all participantsduring prenatal visits at second trimester between 20 to 24 gestational weeks. Maternal ages, weights, and height, gravidity, systolic and diastolic blood pressure, were recorded. Exclusion criteria included twin pregnancy, Women with a history of diabetes; thyroid, liver, or chronic renal disease; or preexisting chronic hypertension (defined as blood pressure >140/90 or need for antihypertensive medications before pregnancy or before 20 wk gestation) also if they had a pregnancy termination, a major anomaly all these cases were excluded or if the pregnancy outcome was unknown (i.e. if they did not deliver at our hospital). Control group were taken from women who entered Mansoura University Hospital, Egypt, Obstetric department, Maternal Study cohort within 2wk of each case and who remained normotensive and non-protein uric throughout pregnancy.. **Ethical approval:** A written informed consent was taken before sample withdrawal. The study was performed in accordance with the ethical standards laid down in Mansoura Faculty of Medicine.

III. METHODS

3ml venous blood samples were withdrowenand allowed to clot between 15℃ and 22℃ for 30 to 90 minutes. Then the samples were centrifuged for 15 minutes at 300 g. The serum were separated ,collected and stored at -20℃ till the time of the assay for PlGF (Human Placenta growth factor, PlGF ELISA Kit /SAB Catalog No: EK1302). Urine Collection: For pregnant women, a midstream clean-catch technique is usually adequate. Ten ml of urine for protein detection by dipstick in at least two random urine specimens obtained at least 4 h apart was required. proteinuria means protein ≥5 gr in a 24 hr (medi-test combi)

RNAextraction from placental tissue:

Fresh samples of 74 human placentas were obtained within 30 min after placental delivery in gynaecological and Obsteric department, Mansoura University, Egypt. After a rinse of the samples with normal saline, the amniotic membranes and the maternal decidua were removed, then the samples were snap frozen in liquid nitrogen and stored at -70C until RNA isolation by using miRNeasy mini kit (Qiagen, cat no. 217004, Germany). RNA was quantified by spectrometry (9).

TaqMan real-time PCR ((provided by Thermo Scientific, U.S.A, cat No. #K1641)Was used to quantify the expression of VEGFgene in placenta.

Reverse Transcription of Extracted RNA to ProducecDNA(10).

One µg (1000 ng) of RNA was reverse-transcribed using Maxima® First Strand cDNA Synthesis Kit provided by Thermo Scientific, USA, cat. no. #K1641. The volume of RNA taken was calculated for each sample separately according to RNA concentration measured by nanodrop. The reaction was done by adding the following to calculated volume of RNA: 4 µl 5X reaction mix (containing the remaining reaction components: reaction buffer, dNTPs, oligo (dT), and random hexamer primers) and 2 µl maxima enzyme mix (containing maxima reverse transcriptase and Thermo Scientific RibolockRNase inhibitor) and the reaction was completed to 20 µl by nuclease-free water. Thus, each 1 µl of the reaction contains 50 ng of RNA. The tubes were incubated for 10 minutes at 25°C followed by 15 minutes at 50°C. The reaction was terminated by heating at 85°C for 5 minutes.

PCR was done using the 2x PCR master-mix solution (i-Taq) provided by iNtRON Biotechnology to check for Tm and product length (11). It was done in a total reaction volume of 20 µl using 10 µl PCR reaction mixture (1X), 1.6 µl template DNA (80 ng), 0.8 µl of 10 µM forward primer (400 nM), 0.8 µl of 10 µM reverse primer (400nM), and 6.8 µl distilled water.

Gene-specific primers were purchased from Invitrogen by Thermo Fisher Scientific. Primer sets for the PCR amplification genes were selected after testing the sequence of the three genes from NCBI database (12). Then, these sequences were submitted in Primer3 tool and checked for product length, melting temperature, GC ratio, self-complementarity, and 3′ complementarity.

The following assays targeting specific mRNAs were included in the study: VEGF: forward5′ -GGGGGCAGAATCATCACGAA-3 and reverse 5-CCAGGGTCTCGATTGGATGG-3.

Quantitative PCR Analysis (13).

Real-time PCR was done for quantification of VEGF gene using SensiFAST SYBR® Lo-ROX (purchased from Bioline, London, UK, catalog number: BIO-94005). For each reaction, the following was used: 10 µl of SensiFAST SYBR Lo-ROX (1X), 0.8 µl of 10 µM forward primer (400nM), 0.8 µl of 10 µM reverse primer (400nM), and 1.6 µl of template (80 ng), and each reaction was completed to reach a total volume of 20 µl by nucleases free water (6.8 µl).

Initial denaturation was done by heating for 1 min at 95°C followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing /extension at 60°C for 30 seconds in 7500 Fast & 7500 Real-Time PCR System (Applied Biosystem, Themo Fisher Scientific, Life Technologies Corporation, USA). Melting curve analysis was done after amplification to confirm the specificity of the product and to exclude the presence of primer–dimers.

The relative gene expression analysis was done by Delta Delta cycle threshold (DDCT) method, and the average DCT of the healthy volunteers for each target gene was used as the calibrator sample (14,15). The amount of target, normalized to an endogenous reference and relative to a calibrator, was calculated. The fold change is obtained by 2^{-DDCT}. This method assigns a value of 0.7 to the calibrator sample, and all other quantities are expressed as an n-fold difference relative to the calibrator.

Statistical analysis

Data analysis was done by Statistical package for social science software (SPSS) version 25. Data are presented as mean ± SD and compared by One-Way ANOVA and they are presented as median (IQR) and compared by Kruskal-Wallis (K-W) test.Significance was considered at p value less than 0.05.

IV. RESULTS

This study involved 74 subjects divided into 3 groups: The first is control group: 24 pregnant ladies with no current or previous pre-eclampsia. Their mean age (years) ± SD = 27.8 ± 4.7 years. The second is mild pre-eclampsia group: 25 pregnant ladies fulfilling the criteria of mild pre-eclampsia.Their mean age±SDwere 27.5 ± 4.3 years. The third is severe pre-eclampsia group: 25 pregnant ladies fulfilling the criteria of severe pre-eclampsia.Their mean age (years) ± SD = 28.8 ± 6.2 years.There was no statistically significant difference between the three study groups as regards age, women weight, gravida and para. It showed that a statistically significant difference existed for the other variables; SBP, DBP, MAP, and fetal weight. Fetal weight was significantly higher in control group than either of the PE groups (P<0.0005). As regards SBP, DBP and MAP, they all were statistically significantly higher in severe PE > Mild PE > Control **(table 1) and (figures 1-7).**There was statistically significant difference between the three study groups as regards 2 studied laboratory variables (PLGF and VEGF). PLGF maternal serum level was statistically significantly higher in control group as compared to either of the two PE groups (p<0.0005) and there is no statistically significantly difference between sPE and mPE but, VFGF was statistically significantly higher in severe PE > Mild PE > Control (P< 0.0005)**(table 2) and (figures 8,9).**There was no statistically significant difference in the proportions of subjects with previous history of PE (P=0.208) and in the starting of PE (P=0.305). There was statistically significant difference in the level of protein in urine with trace and + in mild cases, but ++ and +++ in severe cases (P<0.0005)**(table 3) and figures (10-12).**

V. DISCUSSION

VEGF and its receptors expression within placenta debate, there is substantial discrepancy in the literature concerning levels of placental VEGF in PE pregnancies (7, 8). In 2011, Paiva and colleagues workedon both maternal blood and placental tissue inpreeclampsia and found correlation of gene expression between the two (16). mRNA expressions were, highlyexpressed in preeclampsia, however it have been studied inmaternal blood samples by a few others (17,18,19).**Thepresent study** has analyzed and compared mRNAexpression of VEGF in placenta ofnormotensive and preeclampsia cases. **We found that**, VFGF expression was statistically significantly higher in severe PE > Mild PE > Control(p<0.0005). The present finding supports the results of a number of studies which have reported increasing of VEGF expressionin preeclampsia (20-22). In contrast, some studies have showed an decreased VEGF expression in placental tissues of preeclampsia, Kim et al. reported decreased expressions of VEGF in both level of mRNA and protein in third trimester placental tissue of PE patients (n = 20) compared with an equal number of normotensive controls (p<0.05) (23). Also, Andraweera et al. compared mRNA placental expression of VEGF in placental tissue obtained at deliveryfrom PE (n = 18), gestational hypertension (n = 15) anduncomplicated pregnancy (n = 30). Compared to placentalmRNA from uncomplicated pregnancies, VEGFA werereduced in PE (p = 0.006)(24). A few studies have also reported unaltered expression of VEGF in placental tissues in PE women, Ranheim et al. reported that there were no statistically significant differences in expression of VEGF in mRNA of placental tissues in a study conducted in 25 PE and 19 uneventful pregnancies (25). Also, Sgambati et al. reported that in

cases of PE, the levels of VEGF mRNA were the same as the control group (26).The discrepant results which observed among these studies may be due to phenotypic classification of PE chosen,the differences in sample size, the timing of placental tissue sampling, the method of VEGF quantification, outcome depiction and the references taken for comparison. There are many genetic and environmental factors that may further alter gene expression of preeclampsia. Also, the pathophysiology of VEGF expression in PE is far from complete decipherment with numerous postulates available for explaining different obtained results (22). In this way, we considered it worthwhile to done our study with a case–control model in a different population to add evidence to the existing literature.

A lower PlGF level than normal due to insufficient vascular remodeling of the spiral arteries eventually leads to reduced perfusion of the placenta, which is conceivably involved in the following adverse pregnancy outcomes: SGA, PE, HELLP (hemolysis, increased liver enzymes, and low platelets) syndrome (27,28,29). **Our study** is consistent with many studies which shown that the PlGF level is low in pregnancies complicated by PE (30,31,32).Large studies in different ethnicity in base of VEGF signaling may lead to find predictive marker as well as safe treatment for PE. Early prediction of preeclampesia could have great benefits for prenatal care and early treatment, so attention has been turned toward finding definite non-invasive test. The biomarkers under investigation, angiogenic biomarkers like sFlt1, PlGF and sFlt1 to PlGF ratio are at the most advanced stage. These angiogenic factors correlate with disease severity, could be detected several weeks before clinical presentation of the disease and have predictive value for diagnosis of severe-early onset PE but have a limited capacity in prediction of late onset PE and could not be used alone for intervention, but in combination with other angiogenic factors like soluble endoglin, Doppler sonography and other clinical and biochemical biomarkers they are more useful for predicting severe early onset PE (2).

VI. CONCLUSION

The increase of VEGF gene Expression in placenta tissue and decrease of PLGF level in maternal serum predict the risk of preeclampsia**.**

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Table (1): Comparison of the clinical variables between the three study groups

Variable	Group			Statistic	P value
	Control (n=24)	Mild PE (n=25)	Severe PE (n=25)		
Age (years)	27.8 ± 4.7	27.5 ± 4.3	28.8 ± 6.2	F = 0.459	*0.634
Weight (kg)	82.8 ± 14.8	77.6 ± 13.1	80.3 ± 14.1	F = 0.828	*0.441
Gravida: 1 2 3 4 5	 2 (8.3%) 2 (8.3%) 10 (41.7%) 8 (33.3%) 2 (8.3%)	 0 (0%) 8 (32%) 11 (44%) 6 (24%) 0 (0%)	 1 (4%) 7 (28%) 11 (44%) 5 (20%) 1 (4%)	χ^2 = 8.506	**0.404
Para: 0 1 2 3 4	 4 (16.7%) 5 (20.8%) 9 (37.5%) 5 (20.8%) 1 (4.2%)	 3 (12%) 7 (28%) 11 (44%) 4 (16%) 0 (0%)	 6 (24%) 6 (24%) 9 (36%) 4 (16%) 0 (0%)	χ^2 = 3.830	**0.939
Fetal weight (g)	3125 (3012.5-3237.5) A	2500 (2135-2875) B	2450 (1800-2725) B	K-W = 31.87	***<0.0005
SBP (mmHg)	115 (110-120) A	140 (135-140) B	170 (165-172.5) C	K-W = 61.02	***<0.0005
DBP(mmHg)	75 (75-80) A	85 (80-90) B	110 (107.5-115) C	K-W = 61.66	***<0.0005
MAP(mmHg)	90 (86.6-91.7) A	103 (99.1-105.8) B	130 (126.6-134) C	K-W = 64.24	***<0.0005

***Data are presented as mean ± SD and compared by One-Way ANOVA. **Data are presented as frequency (percentage) and compared by Chi-Square test (Monte Carlo significance). ***Data are presented as median (IQR) and compared by Kruskal-Wallis (K-W) test. Pairwise comparisons are presented as capital letters (similar letters = no significant difference, while different letters = significant difference).**

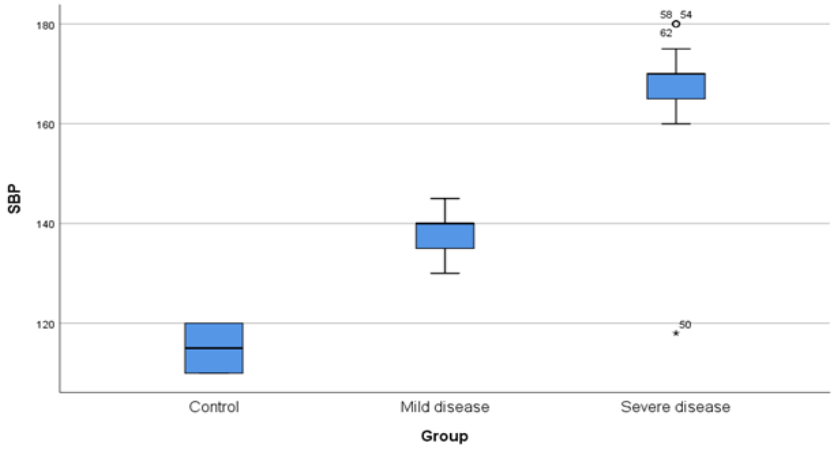

Fig 1: Comparison of SBP between the three study groups

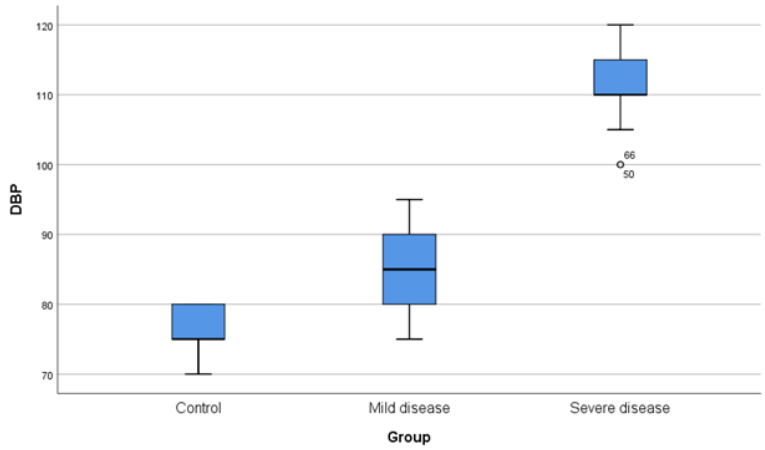

Fig 2: Comparison of DBP between the three study groups

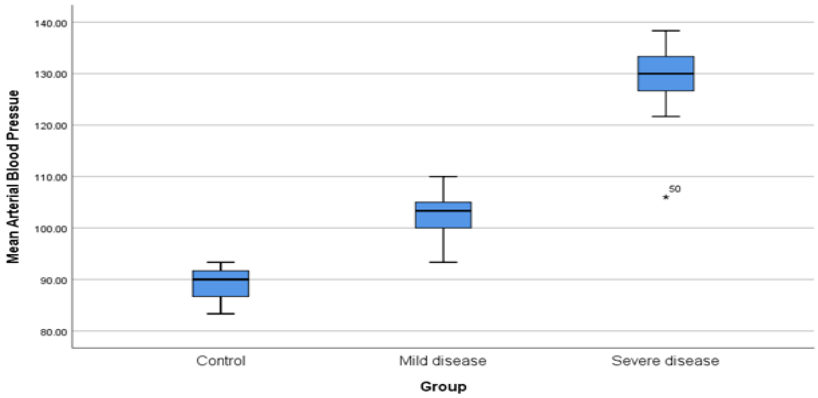

Fig 3: Comparison of ABP between the three study groups

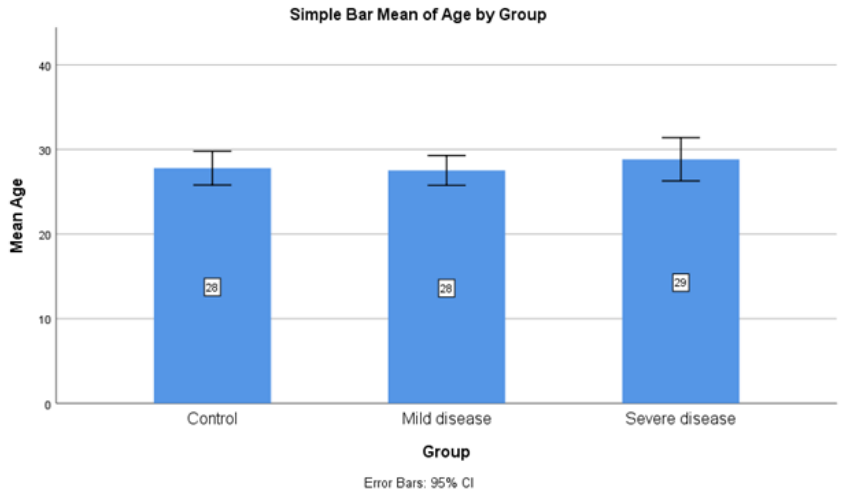

Fig 4: Comparison of mean of age between the three study groups

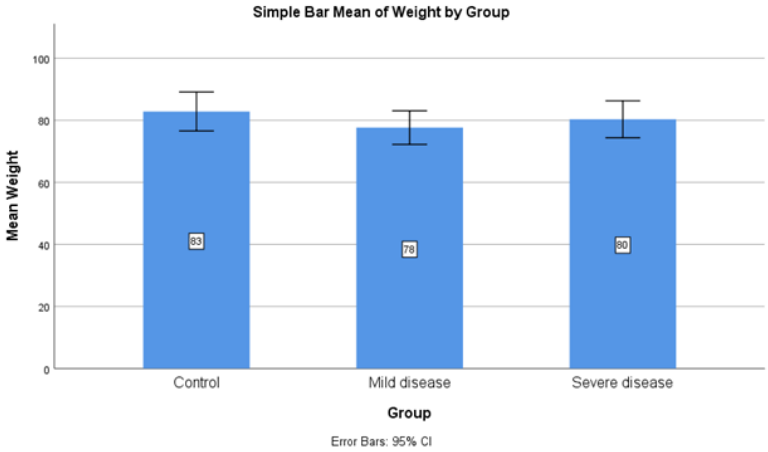

Fig 5: Comparison of mean weight between the three study groups

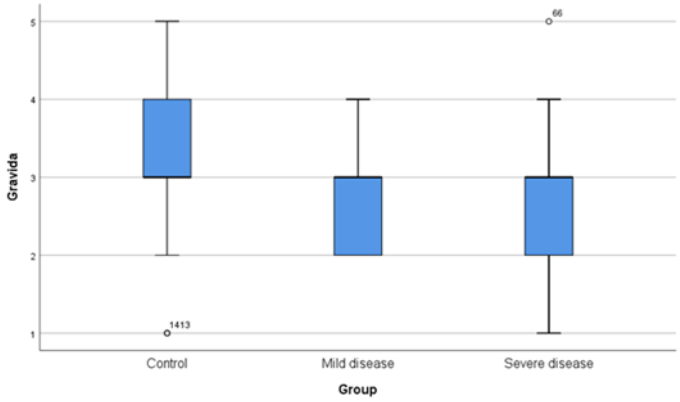

Fig 6: Comparison of gravida between the three study groups

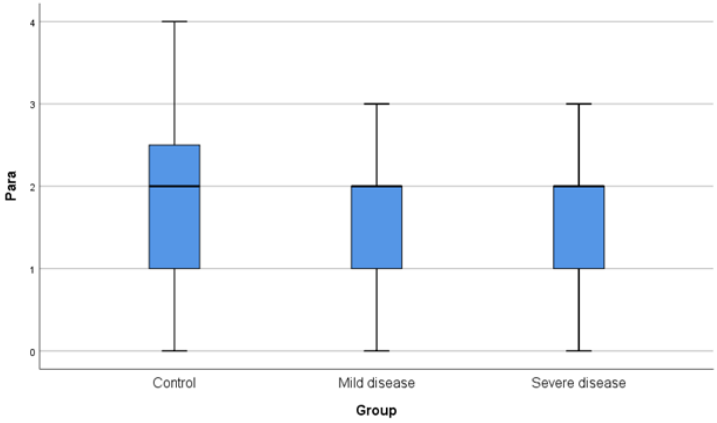

Fig 7: Comparison of Para between the three study groups

Table (2): Comparison of the laboratory variables between the three study groups

Variable	Group			Statistic	P value
	Control (n=24)	Mild PE (n=25)	Severe PE (n=25)		
VFGF	1.3 (1.2-1.37) A	2 (1.9-2.45) B	2.8 (2.55-3.35) C	K-W = 58.07	**<0.0005
PLGF	112.5 (95-180) A	65 (55-80) B	70 (45-85) B	K-W = 30.37	**<0.0005

**Data are presented as median (IQR) and compared by Kruskal-Wallis (K-W) test. Pairwise comparisons are presented as capital letters (similar letters = no significant difference, while different letters = significant difference).

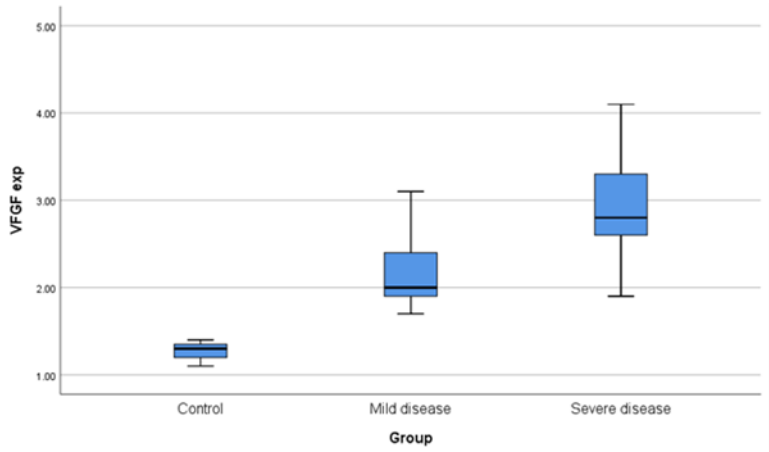

Fig 8: Comparison of VFGF gene expression between the three study groups

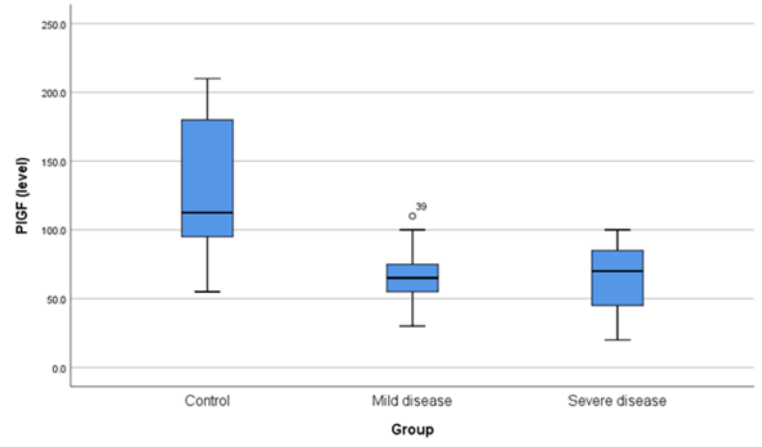

Fig 9: Comparison of PLGF level between the three study groups

Table (3): Comparison of proteinuria and previous PE between mild and severe PE

Variable	Group		Statistic	P value
	Mild PE (n=25)	Severe PE (n=25)		
Protein in urine: Nil Trace + ++ +++	 1 (4%) a 13 (52%) a 11 (44%) a 0 (0%) a 0 (0%) a	 0 (0%) a 0 (0%) b 0 (0%) b 12 (48%) b 13 (52%) b	χ^2=50.000	**<0.0005
Previous PE, Yes	5 (20%)	9 (36%)	χ^2 =1.587	*0.208
Onset of PE (weeks)	30 (27-34)	30 (28-35)	Z =-1.025	***0.305

Data are presented as frequency (percentage) and compared by Chi-Square test* (Monte Carlo significance). Comparison between column proportions was done by Bonferroni adjustments.*****

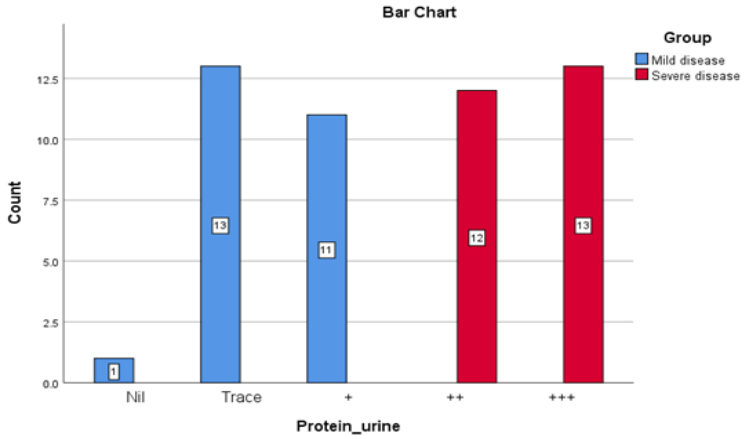

Fig 10: Comparison of proteinuria between mild and severe PE

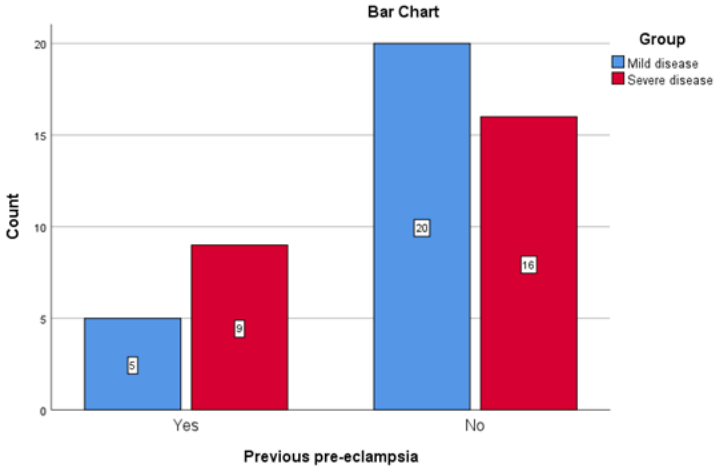

Fig 11: Comparison of previous pre-eclampsia between mild and severe PE groups

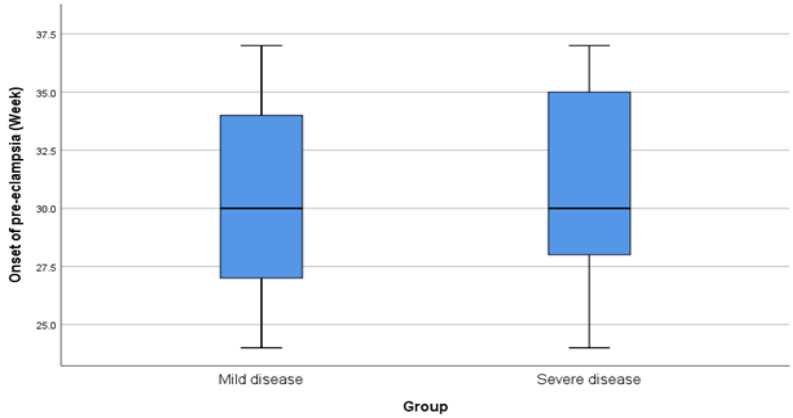

Fig 12: Comparison of onest of pre-preclampsia between mild and severe PE groups

Ibrahim.M.EL-Deen.“ Study of the Vascular Endothelial Growth Factor Gene Expression and Serum Placental Growth Factor Levels in Egyptian Women with Preeclampsia.”IOSR Journal of Pharmacy (IOSRPHR), vol. 9, no. 6, 2019, pp. 28-37.