Fms-Like Tyrosine Kinase-1 (Flt-1) And The Vascular Endothelial Growth Factor (VEGF) As Biomarkers For Associated Preeclampsia

Amal K.Seleem[1] Ibrahim.M.EL-Deen[2], A.A.Abdel Aziz[3],E.H.ELSayd[2], Faeza el Dahtory[4], Ayman z samanodi[5], Ateya G.Amin[6]

ABSTRACT- Vascular endothelial growth factor (VEGF), the major factor for angiogenesis, is necessary for modulating angiogenesis processes in the placenta, fms-like tyrosine kinase 1(Flt-1) is an anti-angiogenic protein that inhibits formation of new blood vessels resulting in potential pregnancy complications. The current study aims to study the placental gene expression of VEGF and (Flt1), genes involved in placental angiogenesis to correlate the degree of angiogenesis imbalance with the degree of preeclampsia. Fifty pre-eclamptic women specimens were collected as follow: 25 women 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 and Flt1 were measured by real time PCR (RTPCR). The mean VEGF 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 Flt1 expression 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 groups. The increase of VEGF and decrease of Flt1 genes expression in placenta tissue predict the risk of preeclampsia.

Keywords: VEGF, Flt-1, *A*ngiogenesis, PE.

1 INTRODUCTION

Abnormal vascular growth and impaired endothelial function in placentas are associated with abnormal pregnancy conditions such as pre-eclampsia. Pre-eclampsia, a life threatening complication of pregnancy, is characterized by the onset of high blood pressure andproteinuria. Pre-eclampsia occurs in about 7–10 % of all pregnancies (1). The pathogenesis of pre-eclampsia is thought to act at three levels, defective placentation, placental ischemia, and endothelial cell dysfunction. Of these, endothelial dysfunction is considered to be a key factor associated with pre-eclampsia (2). The vascular endothelial growth factor (VEGF) family of angiogenic growth factors are important molecules regulating early placental vascular changes. The key molecules VEGF-A and placental growth factor (PlGF) and the receptors VEGF receptor 1 (fms-like-tyrosine-kinase receptor 1,FLT-1) and VEGF receptor 2 (kinase insert domain receptor, KDR) are expressed in the human placenta throughout gestation, and the VEGF family is known to regulate placental angiogenesis and maternal spiral artery remodeling (3,4).

A number of studies have examined the mRNA levels of different angiogenesis regulating factors in placenta from PE patients although results are inconsistent. Some studies report increased VEGF expression (5-6), while others report reduced expression (7,8) in pre-eclamptic women. Other studies found no difference in VEGF expression (9,10). Similarly expression of FLT-1 (11,12) and KDR (9,12,13) have also been examined. Very few studies have simultaneously examined the expression of VEGF and both its receptors in the human placenta in PE (14,15). The majority of these studies evaluate the role of VEGF and its receptors in normal vs PE pregnancy as well as their genetic variations, in predicting these pregnancy complications. The current study aims to study the placental gene expression of VEGF and (Flt1), genes involved in placental angiogenesis to correlate the degree of angiogenesis imbalance with the degree of preeclampsia.

[1]Department of Clinical Biochemistry, Faculty of Medicine , Mansoura University, Mansoura, Egypt.
[2]Department of Chemistry, Faculty of Science, Port Said University, Port Said, Egypt.
[3]Department of Gynecology, Faculty of Medicine, Mansoura University, Mansoura, Egypt.
[4]Department of Genetic, Children Hospital, Mansoura university, Mansoura, Egypt.
[5]Department of Clinical Biochemistry, Faculty of Medicine , Mansoura University, Mansoura, Egypt.
[6]Insurance of health, Central laboratories, Mansoura, Egypt.

*Correspondence
Ateya Gomaa Amin
Telephone: Mobile: 02-01001821713
e-mail: attiagomaa72@yahoo.com

2 Patients AND METHODS

1-Patients:
Fifty patients diagnosed with Preeclampsia were collected from the Obstetrics 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.

2-METHODS :
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).
RNA extraction 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 (16).
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 Produce DNA(17).
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 (18). 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 (19). 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, reverse 5-CCAGGGTCTCGATTGGATGG-3.andFlt1:Forward CTGCAAGATTCAGGCACCTATG, Reverse GTTTCGCAGGAGGTATGGTGCT.
Quantitative PCR Analysis (20).
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 Sensi FAST 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 Bio-system, Thermo 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 cycle threshold (DDCT) method, and the average DCT of the healthy volunteers for each target gene was used as the calibrator sample (21,22). 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.

3 STATISTICAL ANALYSES

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**.**

4 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 Table (1).

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	2 (8.3%)	0 (0%)	1 (4%)		
3	2 (8.3%)	8 (32%)	7 (28%)		
4	10 (41.7%)	11 (44%)	11 (44%)	χ^2 = 8.506	**0.404
5	8 (33.3%)	6 (24%)	5 (20%)		
	2 (8.3%)	0 (0%)	1 (4%)		

*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.

Table 2 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 (2): 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)		
Para:					
0	4 (16.7%)	3 (12%)	6 (24%)		
1	5 (20.8%)	7 (28%)	6 (24%)		
2	9 (37.5%)	11 (44%)	9 (36%)	χ^2 = 3.830	**0.939
3	5 (20.8%)	4 (16%)	4 (16%)		
4	1 (4.2%)	0 (0%)	0 (0%)		
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).

Table 3 showed that a statistically significant difference between the three study groups as regards 2 studied laboratory variables (VEGFand FLT-1). FLT-1level 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, VEGF was statistically significantly higher in severe PE > Mild PE > Control (P< 0.0005).

Table (3): Comparison of the VFGF and FLT-1 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
FLT-1	1.2 (1.1-1.3) A	0.72 (0.64-0.82) B	0.53 (0.42-0.72) B	K-W = 51.35	**<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).

Table 4 showed that 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 (4): 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	1 (4%) a	0 (0%) a		
Trace	13 (52%) a	0 (0%) b	χ^2=50.000	**<0.0005
+	11 (44%) a	0 (0%) b		
++	0 (0%) a	12 (48%) b		
+++	0 (0%) a	13 (52%) b		
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.***

This table showed that there was no statistically significant difference in the proportions of subjects with previous history of PE and in the timing of PE. There was statistically significant difference in the proportions of protein in urine with trace and + in mild cases and ++ and +++ in severe cases.

5 DISCUSION

Vascular endothelial growth factor (VEGF) is a potent regulator of placental vascular function (24). The gene encoding VEGF is highly polymorphic and its functional polymorphisms may be useful as indicators of susceptibility to PE (25). Keshavarzi et al. found that The relative mRNA expression of VEGF gene in placental tissue was significantly higher in PE women compared to controls (35). Also, Chung et al. showed that total VEGF mRNA expression was increased 2.8-fold(p\0.05) in PE versus normal placenta (25). The present study, is agree with both of Keshavarzi et al. and Chung et al., it has analyzed and compared mRNA expression of VEGF in placenta of normo-tensive and preeclampsia cases. We found that, VEGF expression was statistically significantly higher in severe PE > Mild PE > Control(p<0.0005). Also, the present finding supports the results of a number of studies which have reported increasing of VEGF expression in preeclampsia (26,27).

In contrast, some studies have showed an decreased VEGF expression in placental tissues of preeclampsia, Cooper et al found that levels of VEGF mRNA were significantly lower in the pre-eclamptic women compared with the control women (28). Andraweera et al. (15) compared mRNA placental expression of VEGFA in placental tissue obtained at delivery from PE (n = 18), gestational hypertension (n = 15) and uncomplicated pregnancy (n = 30). Compared to placental mRNA from ncomplicated pregnancies, VEGFA were reduced in PE (p = 0.006) andgestational hypertension (p\0.001) placentae (15). Kim et al also reported decreased expressions of VEGF in both level of mRNA and protein in placenta of preeclamptic patients compared with the normotensive controls(8) .

A few studies have also reported unaltered expression of VEGFA in placental tissues in PE women (29, 30). Sgambati et al reported that in the cases of preeclampsia, the levels of VEGF mRNA were the same as the control group (31). Ranheim et al. reported that there were no statistically significant differences in expression of VEGF in mRNA levels for either the decidua basalis or placental tissues in a study conducted in 25 PE and 19 uneventful pregnancies (32).

Flt1 gene produces two mRNAs in placenta and vascular endothelial cells, a long form for the full-length receptor Flt1 and a short form for sFlt1 which carries only the ligand binding region (33) . Toft *et al* studied on the transcriptomes of placental tissues from PE and small for gestational age (SGA) pregnancies by whole-genome microarray and quantitative Real time PCR.it showed that increased expression of Flt1 was detected by QRT-PCR in the PE +SGA group but microarray analysis did not reveal any significant differences between groups (9). But, Marni showed that no clear evidence of an association between sFlt-1 levels in first trimester and adverse pregnancy outcomes (36).

The present study has analyzed and compared mRNA expression of Flt1 in placenta of normotensive and preeclampsia cases and our findings revealed that, Flt1 gene Expression in placenta tissue 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 two groups of pre-eclamps.

Results inconsistance of these studies may be due to the differences in phenotypic classification of PE chosen sample size, phenotypic classification of PE chosen, the timing of placental tissue sampling, the method of VEGF quantification and out comedepiction and the references taken for comparison (37,38).There are many genetic and environmental factors that may further alter gene expression of preeclampsia. Also, the patho-physiology of VEGF expression in PE is far from complete decipherment with numerous postulates available for explaining different obtained results (30, 38), However, findings were affected by ethodological, biological and testing variations between studies; highlighting the need for consistent testing of new biomarkers and reporting of outcome measures.

6 Conclusion:

The increase of VEGF and decrease of Flt1 gene Expression in placenta tissue predict the risk of preeclampsia.

7 REFERENCES

1. August, P.; Lindheimer, MD. Pathophysiology of preeclampsia. In: Laragh, JL.; Brenner, BM., editors. Hypertension. 2. New York: Raven Press; 1995. p. 2407-2426.

2. Roberts JM, Lain KY. Recent Insights into the pathogenesis of pre-eclampsia. Placenta. 2002; 23:359–372. [PubMed: 12061851].
3. Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Peruzzi CA,Detmar M. Stimulation of endothelial cell migration by vascular permeabilityfactor/vascular endothelial growth factor through cooperative mechanismsinvolving the alphavbeta3 integrin, osteopontin, and thrombin. Am J Pathol1996;149:293–305.
4. Charnock-Jones DS, Kaufmann P, Mayhew TM. Aspects of human fetoplacentalvasculogenesis and angiogenesis(2004). Molecular regulation. Placenta 2004;25:103–113.
5. Kumazaki K, Nakayama M, Suehara N, Wada Y: Expression of vascular endothelial growth factor, placental growth factor, and their receptorsFlt-1 and KDR in human placenta under pathologic conditions.Hum Pathol 2002, 33:1069–1077.
6. Kweider N, Fragoulis A, Rosen C, Pecks U, Rath W, Pufe T, Wruck CJ:Interplay between vascular endothelial growth factor (VEGF) and nuclearfactor erythroid 2-related factor-2 (Nrf2): implications for preeclampsia.J Biol Chem 2011, 286:423863–423872.
7. Lyall F, Young A, Boswell F, Kingdom JC, Greer IA: Placental expression of vascular endothelial growth factor in placentae from pregnancies complicated by pre-eclampsia and intrauterine growth restriction does not support placental hypoxia at delivery. Placenta 1997, 18:269–276.
8. Kim SC, Park MJ, Joo BS, Joo JK, Suh DS, Lee KS: Decreased expressions of vascular endothelial growth factor and visfatin in the placental bed of pregnancies complicated by preeclampsia. J Obstet Gynaecol Res 2012, 38:665–673.
9. Toft JH, Lian IA, Tarca AL, Erez O, Espinoza J, Eide IP, Bjørge L, Draghici S, Romero R, Austgulen R: Whole-genome microarray and targeted analysis of angiogenesis regulating gene expression (ENG, FLT1, VEGF, PlGF) in placentas from pre-eclamptic and small-for-gestational-age pregnancies J Matern Fetal Neonatal Med 2008, 21:267–273.
10. Sgambati E, Marini M, Zappoli Thyrion GD, Parretti E, Mello G, Orlando C, Simi L, Tricarico C, Gheri G, Brizzi E: VEGF expression in the placenta from pregnancies complicated by hypertensive disorders. Br J Obstet Gynaecol 2004, 111:564–570.
11. Heydarian M, McCaffrey T, Florea L, Yang Z, Ross MM, Zhou W, Maynard SE:Novel splice variants of sFlt1 are upregulated in preeclampsia. Placenta2009, 30:250–255.
12. Munaut C, Lorquet S, Pequeux C, Coulon C, Le Goarant J, Chantraine F,Noël A, Goffin F, Tsatsaris V, Subtil D, Foidart JM: Differential expressionof Vegfr-2 and its soluble form in preeclampsia. PLoS One 2012,7:e33475.
13. Tripathi R, Rath G, Jain A, Salhan S: Soluble and membranous vascularendothelial growth factor receptor-1 in pregnancies complicated bypre-eclampsia. Ann Anat 2008, 90:477–489.
14. Helske S, Vuorela P, Carpén O, Hornig C, Weich H, Halmesmäki E: Expression of vascular endothelial growth factor receptors 1, 2 and 3 in placentas from normal and complicated pregnancies. Mol Hum Reprod 2001, 7:205–210.
15. Andraweera PH, Dekker GA, Laurence JA, Roberts CT: Placental expressionof VEGF family mRNA in adverse pregnancy outcomes. Placenta 2012,33:467–472.
16. E. Macfarlane and C. E. Dahle, ―Isolating RNA from whole blood--the dawn of RNA-based diagnosis?‖ Nature, 1993. vol. 362, no. 6416, pp. 186–188.
17. J. Sambrook, E. F. Fritschi, and T. Maniatis, Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, New York, NY, USA, 1989.
18. W. M. Freeman, S. J. Walker, and K. E. Vrana, ―Quantitative RT-PCR: pitfalls and potential,‖ Biotechniques, 1999 vol. 26, no. 1, pp. 112–122.
19. R. K. Saiki, D. H. Gelfand, S. Stoffel et al., ―Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase,‖ Science,1988; vol. 239, no. 4839, pp. 487–491.
20. J. Ye, G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. Madden, ―Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction,‖ BMC Bioinformatics,2012; vol. 13, article 134.
21. R. Higuchi, C. Fockler, G. Dollinger, and R. Watson, ―Kinetic PCR analysis: real-time monitoring of DNA amplification reactions,‖ Nature Biotechnology,1993; vol. 11, no. 9, pp. 1026–1030.
22. K. J. Livak and T. D. Schmittgen, ―Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method,‖ Methods,2001; vol. 25, no. 4, pp. 402–408.
23. Valquiria Maria de Paula Cunha I ; Roseane Lopes da Silva Grecco II ; Marina Carvalho Paschoini III ; Sueli Riul da Silva VI ; Mariângela Torreglosa Ruiz V ; Marly Aparecida Spadotto Balarin. Genetic polymorphisms of vascular endothelial growth factor in pre-eclampsia Rev.(2011). Bras. Ginecol. Obstet. Vol.33 no.7.
24.Chung, Soo-Cheon Chae. Interleukin-27 polymorphisms areassociated with inflammatory bowel diseases in a Korean population. (2009). Volume 24, Issue 10 October 2009 Pages 1692–1696.
25. Valquiria Maria de Paula Cunha I ; Roseane Lopes da Silva Grecco II ; Marina Carvalho Paschoini III ; Sueli Riul da Silva VI ; Mariângela Torreglosa Ruiz V ; Marly Aparecida Spadotto Balarin . Genetic polymorphisms of vascular endothelial growth

factor in pre-eclampsia Rev. Bras. Ginecol. Obstet. vol.33 no.7 Rio de Janeiro July 2011.
26. Deepali P Sundrani1, Umakar S Reddy3, Asmita A Joshi. Differential placental methylation and expression of VEGF, FLT-1 and KDR genes in human term and preterm preeclampsia. Clinical Epigenetics. 2013, 5:6.doi:10.1186/1868-7083-5-6.
27. RachnaAgarwal, NeelamKumari, RajarshiKar, Nilesh Chandra, ArchanaNimesh. Evaluation of Placental VEGFA mRNA Expressionin Preeclampsia: A Case Control Study. Federation of Obstetric & Gynecological Societies of India. 2018; 68(2): 88–95.
28. Cooper JC, Sharkey AM, Charnock-Jones DS, Palmer CR, Smith SK. VEGF mRNA levels in placentae from pregnancies complicated by pre-eclampsia. *BJOG* 1996; 103: 1191-1196.
29. Staff AC, Benton SJ, von Dadelszen P, et al. Redefining preeclampsia using placenta-derived biomarkers. Hypertension. 2013;61:932–42.
30. Jim B, Karumanchi SA. Preeclampsia: pathogenesis, prevention, and long-term complications. Semin Nephrol. 2017;37:386–97.
31. Sgambati E, Marini M, Zappoli Thyrion GD, Parretti E, Mello G, Orlando C, et al. VEGF expression in the placenta from pregnancies complicated by hypertensive disorders. BJOG 2004; 111: 564-570.
32. Ranheim T, Staff AC, Henriksen T. VEGF mRNA is unaltered in decidual and placental tissues in preeclampsia at delivery. *Acta Obstet Gynecol Scand* 2001; 80: 93-98.
33. Levine RJ, Maynard SE, Qian C, Lim K-H, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. *New Engl J Med* 2004; 350: 672-683.
34. Maynard SE, Min J-Y, Merchan J, Lim K-H, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003; 111: 649-658.
35. Keshavarzi, F., Shahrakipoor, M., Teimoori, B., Yaghmaei, M., Narooei-Nejad, M., Rasooli, A., & Salimi, S.. Association of the placental VEGFpromoter polymorphisms and VEGF mRNA expression with preeclampsia.(2018). *Clinicaland Experimental Hypertension*, 1-6.
36. Marni Jacobs, Natasha Nassar, Christine L Roberts1,2, Ruth Hadfield1 , Jonathan M Morris1,2 and Anthony W Ashton.(2011).Levels of soluble fms-like tyrosine kinase one in first trimester and outcomes of pregnancy: a systematic review Jacobs et al. Reproductive Biology and Endocrinology 2011, 9:77.
37. Nikuei P, Malekzadeh K, Rajaei M, et al. The imbalance in expression of angiogenic and anti-angiogenic factors as candidatepredictive biomarker in preeclampsia. Iran J Reprod Med. 2015;13:251–62.
38. Andraweera PH, Dekker GA, Roberts CT. The vascular endothelial growth factor family in adverse pregnancy outcomes. Hum Reprod Update. 2012;18:436–57.