Original Investigations

Analyses of soluble endoglin and matrix metalloproteinase 14 using enzyme-linked immunosorbent assay in the diagnosis and/or the determination of the severity of late/early-onset preeclampsia
Ovayolu et al. MMP-14, endoglin and preeclampsia

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DOI: 10.4274/jtgga.galenos.2020.2019.0201

Received: 18 April, 2020**Accepted:** 01 July, 2020

Abstract
Objective: Abnormal trophoblastic invasion and impaired placentation have a crucial role in the etiopathogenesis of preeclampsia (PrE). Trophoblastic cells produce invasion into the maternal decidua and create remodelling in the spiral arteries with matrix metalloproteinase-14 (MMP-14). MMP-14 cleavage on endoglin releases its extracellular part into the maternal circulation to form soluble form of endoglin (s-ENG). In PrE, There is a relationship between endothelial dysfunction and s-ENG. So, our study aimed to determine the levels of serum s-ENG and MMP-14 in patients with PrE and healthy subjects.
Material and Methods: The study included 30 patients with late-onset preeclampsia (L-PrE) as group 1 (gestational age≥34 weeks), 33 patients with normal pregnant women as group 2 (gestational age≥34 weeks), 31 patients early-onset preeclampsia (E-PrE) as group 3 (gestational age<34 weeks), and 31 patients with normal pregnant women as group 4 (gestational age<34 weeks). s-ENG and MMP-14 concentrations measured using enzyme-linked immunosorbent assays were compared.
Results: In all pregnant women, MMP-14 concentrations were found to decrease with increasing gestational age. s-ENG concentrations were highest in the E-PrE group. Amongst the patients belonging to groups 1 and 3, 29 had mild PrE while 32 suffered severe PrE. There was no significant difference between s-ENG concentrations in mild and severe preeclamptic women (p=.133), but there was a significant difference between MMP-14 concentrations in

these pregnant women (3.11±0.61, 3.54±1.00, p=.047, respectively). There was no correlation between s-ENG and MMP-14 concentrations (p>.05).
Conclusion: MMP-14 and s-ENG concentrations can be predictive biomarkers in the diagnosis of PrE, and maternal serum MMP-14 concentration can be also measured as a biomarker in determining the severity of PrE.
Keywords: endothelial dysfunction; hypertension; implantation; severe pre-eclampsia; trophoblast

Introduction

Preeclampsia (PrE) is still one of the important obstetric syndromes. The main features include protein leakage in urine and new-onset gestational hypertensive disease, or other signs/symptoms of PrE in the absence of proteinuria after 20 weeks' gestation. This still remains important for maternal/infant at morbidity/mortality worldwide (1). Although improvements have been reported in the diagnosis and treatment of PrE in previous studies, there are still many questions that await answers. In PrE, the disabled capacity of the extravillous trophoblasts (EVTs) for invasion into the spiral arteries causes an inadequately perfused fetal-placental unit. When the embryo is implanted, EVTs invade the decidua and remodel spiral arteries getting until the inner third of the myometrium. Matrix metalloproteinases (MMPs) enable the infiltration of EVTs to the uterine wall. Decidual stromal cells produce high concentrations of MMPs, boosting EVTs invasive potential (2, 3). The dysregulation of MMPs causes inadequate trophoblast invasion, inadequate uterine and spiral artery remodeling, and finally causes the development PrE (4, 5).
MMPs are calcium and also zinc-dependent proteases that break down different components of the extracellular matrix (ECM). MMPs are key to the mediation of apoptosis, cell proliferation, cell–cell adhesion, cell migration and invasion, and tissue remodeling (6). MMP-14 was stained in the membrane of trophoblast and vascular endothelial cells (7). In a normal pregnancy, a notable increase was detected in MMP-14 in the last trimester versus the first trimester (8). In PrE, the abnormal release of vasoactive factors such as MMP-1 and MMP-14 occurs near the end of pregnancy, thereby contributing to the development of hypertension. MMP-1 and MMP-14 were also investigated in other obstetric syndromes such as premature rupture of membranes and preterm labor. MMPs have been investigated in inflammation, malignant growth, as well as reproductive and neurologic disorders (2). Further, analysis of gene expression demonstrated that both MMP-14 and endoglin gene expression was raised in PrE (9).
Endoglin (CD105) is an integral membrane-bound glycoprotein and it is also a transforming growth factor-beta co-receptor. High endoglin concentrations on the syncytiotrophoblasts in patients with severe PrE were shown with western blot analysis and immunohistochemistry staining. A soluble form of endoglin (s-ENG) has been demonstrated in human blood. s-ENG is released in endothelial tissues, phagocytes, syncytiotrophoblasts, and smooth muscle cells. The role and direct molecular mechanism of s-ENG is not clear; however, it is capable of reducing angiogenesis (10, 11). MMP-14 cleavage on endoglin releases its extracellular part into the maternal circulation to form s-ENG. The rise in s-ENG expression in PrE is proportional to the severity of the disease and reduces after delivery. The relationship between endothelial dysfunction following poor placentation and s-ENG has also been shown in PrE. Several studies have shown that MMP-14 has importance in the reduction of the s-ENG concentrations for the alleviation of the clinical manifestations of PrE (2, 8).
A test/method that predicts PrE or confirms its diagnosis before its clinical occurrence has not yet been found. Many studies have been conducted on PrE and MMP-14. However, there are no studies on the measurement of serum MMP-14 concentrations using enzyme-linked

immunosorbent assay (ELISA) in PrE, which is non-invasive, easy, fast, and inexpensive. Our study aimed to evaluate the values of maternal serum MMP-14 and s-ENG in patients with PrE, and also to address their relationship with PrE severity.

Material and Methods

Patient selection

Our subjects were included from Cengiz Gokcek Public Hospital, Gaziantep, Turkey at the department of obstetrics and gynecology between January 2018 and December 2018. This prospective study was conducted according to the Declaration of Helsinki, and the institutional ethical review board of Gaziantep University approved the study (acceptance no: 2018/91). A total of 138 pregnant women were recruited to the study out of which 13 were excluded on grounds of incomplete fetomaternal details, refusal to participate in the study, and an SGA fetus in the control group (Figure 1). Out of these 125 women, 30 with late-onset preeclamptic (L-PrE) at ≥34 weeks of gestation were constituted as group 1, 33 healthy women with ≥34 weeks constituted group 2, 31 women with early-onset preeclampsia (E-PrE) at <34 weeks gestation comprised group 3 and 31 healthy women with <34 weeks pregnancy constituted group 4. Groups 2 and 4 were maternal age and gestational age-matched controls for groups 1 and 3 respectively. The control groups comprised healthy pregnancies who presented to our hospital for routine obstetric examinations. All subjects were informed about the study and each gave written consent. Gestational age assessment is based concerning the last menstrual time or first-trimester ultrasonography of obstetric measurements.

The diagnosis of PrE was accepted based on the existence of proteinuria (urinary excretion of protein ≥300 mg in a 24-hour urine specimen, or proteinuria ≥1+ in dipstick) and a maternal blood pressure level of ≥90/140 mm Hg (two blood pressure measurements 6 h apart), occurring after 20 weeks of gestation in a previously normotensive woman, as defined by the Committee on Terminology of the American College of Obstetricians and Gynecologists (ACOG). Until the diastolic and/or systolic blood pressure reached 110/160 mm Hg, it was accepted as mild, and if these values exceeded this level, it was accepted as severe (12). Small for gestational age (SGA) newborns were accepted as birth weight <10th percentile for gestational age with Turkey's national nomogram as the reference for fetal growth (13). Maternal body mass index (BMI) (kg/m^2) was accounted as the ratio of the maternal weight (kg) to the square of the maternal height (m). The exclusion criteria for both groups were as follows: pregnant women with any systemic disease (e.g. chronic hypertension, diabetes mellitus, thyroid diseases, liver and kidney diseases), using any kind of medication throughout pregnancy, any medication use for PrE treatment at the time of first admission, pregnancies complicated with premature rupture of membranes or chorioamnionitis, pregnant women who had fever at the time of first admission, patients who had fetal congenital abnormalities or genetic syndromes, smoking, multiple gestation and active labor.

Each pregnant woman had obstetric ultrasound examinations and fetal/maternal assessments, which were conducted by a single obstetrician-gynecologist specialist (A.O.). Obstetric anamneses were obtained from all pregnant women. Demographic data such as age, gravidity, parity, BMI, and gestational age were recorded. Maternal venous blood samples were taken for measurement of s-ENG and MMP-14 concentrations after the diagnosis of PrE in the outpatient clinic. The samples were immediately centrifuged at 1500 *g* for 10 min, and serum samples were separated and stored at -80°C until required for analysis. All patients with E-PrE were hospitalized. After the hospitalization, a betamethasone injection was administered without delay. Pregnancy was terminated immediately in the event of urgent fetal/maternal situations such as severe PrE development or fetal distress. Otherwise, maternal blood pressure was measured frequently (at least every 4 hours) during rest and the arm held at the level of the heart. Hypertension can be persisted within a shorter interval in patients with

diastolic and/or systolic blood pressure ≥110 mmHg and ≥160 mmHg, respectively, to facilitate timely antihypertensive treatment. In cases of E-PrE, delivery should be delayed for at least 48 hours if maternal and fetal status permit. During this period, betamethasone injections for lung maturation (two doses of 12 mg at 24-hour intervals) were administered. All patients with L-PrE were also hospitalized and their pregnancies were terminated. Pregnant women with uncomplicated pregnancies were randomly selected at the same time as the case selection was performed to serve as controls. The samples of the control groups were obtained during routine obstetric examinations in the last trimester of pregnancy. These women were then followed up till delivery. The four groups were compared in terms of maternal age, BMI, gravida, parity, week of gestation, systolic/diastolic blood pressure, full blood count, liver function tests (AST, ALT), blood urea nitrogen, creatinine, s-ENG, MMP-14, total protein in spot urine sample, and infant weight at delivery.

Serum MMP-14 and s-ENG analysis

MMP-14 concentrations were also assessed using a commercial ELISA kit, the Human MMP-14 ELISA Kit (Rel Assay Diagnostics, Gaziantep, Turkey), in accordance with the manufacturer's instructions. The human MMP-14 ELISA kit is based on the principle of biotin double-antibody sandwich technology. s-ENG concentrations were assessed using a commercial ELISA kit that was specific for the detection of human s-ENG with high sensitivity and specificity (Rel Assay Diagnostics, Gaziantep, Turkey). The measurements were taken in line with manufacturer's instructions. The kit uses the sandwich-ELISA principle.

Statistical analysis

Statistical analyses were performed using the SPSS for Windows version 25.0 software package, and p values <0.05 were accepted as statistically significant (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). In the analyses of the variables, the Student's t-test was used for comparing two groups. One-way analysis of variance (ANOVA) was used for the comparison of four groups. In addition, the demographic data of the variables were determined using frequency analyses.

Results

A total of 61 pregnant women with PrE were included in our study. The control groups included 64 healthy pregnant women. The demographic data of the groups were compared (Table 1). There was no significant difference between maternal age, gravidity, and parity ($p > .05$), but there was a significant difference between BMI, gestational age, systolic blood pressure, diastolic blood pressure, and birth weight ($p<.05$). The laboratory parameters of the study and control groups are shown in Table 2. MMP-14 and s-ENG concentrations differed between the groups, as shown in Table 2 and Figures 2, 3. MMP-14 concentrations were the same in group 1 and group 2, and also in group 3 and group 4. Briefly, there were no differences between the PrE groups and their control groups. The difference for MMP-14 was significant in pregnant women who were between <34 weeks and ≥34 weeks' gestation. In other words, in all pregnant women, as the gestational age increased, MMP-14 concentrations in the maternal serum were observed to decrease.

We determined the s-ENG concentrations at the highest level in the E-PrE group. No differences were detected between s-ENG and MMP-14 concentrations in mild (21 patients) and severe PrE (9 patients) in the L-PrE group ($p=.829$, $p=.210$, respectively). No differences were detected between s-ENG and MMP-14 concentrations in mild (8 patients) and severe PrE (23 patients) in the E-PrE group ($p=.887$, $p=.739$, respectively). No differences were detected between s-ENG concentrations in mild (29 patients) and severe PrE (32 patients) in the PrE groups (group 1 and group 3) ($p=.133$); however, there was a significant difference between the MMP-14 concentrations (3.11±0.61, 3.54±1.00, $p=.047$, respectively) (Table 3).

When the patients with and without SGA infants (10 patients and 115 patients, respectively) were compared, it was determined that there were no differences between the s-ENG and MMP-14 concentrations (p=.133, p=.969, respectively). When the patients with and without new-onset cerebral or visual disturbances (15 patients and 110 patients, respectively) were compared, there were no differences between s-ENG and MMP-14 concentrations (p=.528, p=.573, respectively). When the patients with and without right upper quadrant or epigastric pain (8 patients and 117 patients, respectively) were compared, it was determined that there were no differences between the s-ENG and MMP-14 concentrations (p=.162, p=.154, respectively). When those who had first gravidity (31 patients) and gravidity >1 (94 patients) were compared, no differences were found between the s-ENG and MMP-14 concentrations (p=.855, p=.364, respectively). When those whose BMI scores were <30 kg/m^2 (56 patients) and ≥30 kg/m^2 (69 patients) were compared, there were no differences between the s-ENG and MMP-14 concentrations (p=.373, p=.873, respectively). When those whose maternal age was <35 years (100 patients) and ≥35 years (25 patients) were compared, no differences were found between s-ENG and MMP-14 concentrations (p=.167, p=.625, respectively). No statistically significant relation was detected between s-ENG and MMP-14 concentrations (p>.05).

Discussion

In the present study, the maternal blood concentrations of s-ENG and MMP-14 were evaluated in order to examine the association between diagnoses of L-PrE/E-PrE, the severity of PrE, and these biomarkers. We found that serum s-ENG and MMP-14 concentrations were different in the four groups. The concentrations of s-ENG were at the highest in the E-PrE group (group 3) in which the severe PrE was at the highest level. MMP-14 concentrations, on the other hand, were found to be highest in severe PrE cases.

The contact between the placenta and the decidua ensures the communication between the fetus and the mother. The trophoblast cells in the embryo initially attach to the uterine epithelium. The trophoblasts differentiate into EVTs, which degrade the uterine epithelium basement membrane and ECM, then migrate into the decidual stroma. EVTs are characterized by their invasiveness, ensuring sufficient contact with the maternal circulation. This invasion stage is under strict control, restricted to the decidua and the proximal third of the myometrium in healthy pregnancies. The limitation of this invasion is related to fetal growth restriction and PrE. Invasive processes are generally assisted by the expression and activity of MMPs (8). EVTs also reach and remodel the spiral arteries, transforming them into low resistance vessels, a process necessary to allow an adequate blood supply to the fetus. It was shown in previous studies that this process occurs with the MMP-14 and MMP-15 regulation through the mediation of endotelin-1 in a low oxygen environment (2-3% O_2) (14). These pathologic changes constitute a process that starts in the first trimester, proceeds with a normal pregnancy period, and is finally reflected to the physician with an excessive clinical manifestation in the last trimester. In actual fact, these events consist of pathogenic factors that are produced in the placenta as a response to hypoxia, mixing with the maternal blood causing endothelial dysfunction. Despite the excessively excreted molecules, the inflammatory factors or those that are antiangiogenic and autoimmune are far more important (15).

Several authors demonstrated that the ECM and MMP-14 were vital regulators of angiogenesis. Briefly stated, placental MMPs may influence the spiral artery remodeling in implantation. In PrE, MMPs have roles in prompting vasoconstriction, changes in vascular reactivity, and endothelial harm (16, 17). In light of this knowledge, the imbalance of angiogenic and/or anti-angiogenic factors in PrE, including endoglin/s-ENG and MMP-14, represents one potential mechanism that has gained significant attention recently. On the other

hand, MMP-14's expression position and distribution were in accordance with endoglin's expression and location. To state this more specifically, MMPs have become important biomarkers in the identification of women with an elevated risk of developing PrE and also important biologic targets for treating this syndrome (1, 7, 18). When MMP-14 measurements are investigated in tissues after the delivery, this is a multi-stage, laborious and expensive procedure. Whereas, we planned to evaluate MMP-14 through direct measurements of maternal serum, which can be measured in ongoing pregnancy. But, the serum concentrations of MMP-14 may not directly reflect its overall expression in PrE because of its significant membrane-binding properties (19).

Zhang et al. found that s-ENG concentrations were high in patients with severe PrE, and showed that this event was triggered by MMP-14 in which lead to the development of severe PrE (7). Levine et al., on the contrary, reported that there were no important differences in terms of s-ENG concentrations between women with mild PrE and severe PrE, recommending that no correlation was found between s-ENG concentrations and the severity of PrE. However, it was also shown that the obvious increase in s-ENG started 2-3 months before the onset of PrE. They also reported that this increase was greater in E-PrE than in L-PrE (9, 20). Sezer et al. conducted a study on a PrE group comprising patients with E-PrE and L-PrE and found that s-ENG concentrations were high in the maternal circulation and also umbilical cord blood (4). Zafer et al. compared the s-ENG concentrations in maternal serum with amniotic fluid s-ENG concentrations and reported that they were incompatible (11). All these findings suggest that all compartments have independent dynamics in terms of s-ENG. Moreover, MMP-14 may have independent dynamics, like s-ENG. When all patients with severe PrE were analyzed, MMP-14 concentrations were found to be higher although s-ENG was not different. Interestingly, this made us consider that MMP-14 serum concentrations in PrE may be more significant than s-ENG. Although s-ENG and MMP-14 concentrations showed a similar pattern, the relationship between the two showed no statistically significant correlation. This may be owing to the membrane-binding properties of MMP-14.

We acknowledge strengths and limitations of the study. The most important limitation of our study is the small number of participants. We do not have the pre-pregnancy BMI of all participants. s-ENG and MMP-14 concentrations could have been examined in different compartments such as the placenta, umbilical cord serum, and amniotic fluid. The other limitation is that there are no uterine artery Doppler studies in this study. The strengths of the study were that none of the patients had any treatments for PrE and only participants who were not at active labor were selected for the study. Another strength is the comparison of MMP-14 serum concentrations with s-ENG, which has a major role in angiogenesis and endothelial cell function.

Conclusion

Larger basic and clinical studies, as well as meta-analysis should be performed to assess whether MMPs have an important role in the pathophysiology of PrE and the predictive value of these enzymes as biomarkers or therapeutic targets. Finally, in view of the literature data, we believe that testing s-ENG is worthy of further investigation for the early diagnosis of PrE. In addition, it may be logical to investigate serum MMP-14 concentrations in predicting patients who will proceed to a severe form of PrE.

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

Footnote: This manuscript was presented as an oral presentation at the Maternal Fetal Medicine and Perinatology Society of Turkey 2019 Perinatal Medicine Congress, May 9th-11th, 2019, at the Hilton Hotel, Izmir, Turkey.

Acknowledgments: We would like to thank Assoc. Prof. Dr. İsmet Gün from Sultan Abdulhamid Han Sample Training and Research Hospital, Istanbul, Turkey for providing insight, expertise, and scientific guidance that greatly assisted our research.

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Table 1. The demographicdata of the groups

Variables	L-PrE group 1 (n=30)	Control group 2 (n=33)	E-PrE group 3 (n=31)	Control group 4 (n=31)	p
Age (years, mean±SD)	28.9±6.4	25.8±6.0	29.1±6.6	28.9±6.6	.122
BMI (kg/m^2, mean±SD)	32.2±5.4^{a}	28.6±4.9^{b}	31.3±5.0^{ab}	29.5±4.7^{ab}	**.020**
Gestational age (weeks, mean±SD)	37.2±1.5^{a}	37.7±1.5^{a}	31.1±2.2^{b}	30.5±2.0^{b}	**.001**
Gravidity (mean±SD)	3.3±2.4	3.5±5.4	3.1±1.7	3.7±2.3	.887
Parity (mean±SD)	1.9±1.9	1.5±1.3	1.5±1.4	1.9±1.7	.620
Syst TA (mm/Hg, mean±SD)	160±18^{b}	105±10^{c}	173±18^{a}	102±10^{c}	**.001**
Diast TA (mm/Hg, mean±SD)	103±10^{b}	65±6^{c}	110±11^{a}	65±7^{c}	**.001**
Birth weight (gram, mean±SD)	2960±691^{a}	3249±483^{a}	1650±467^{b}	3172±346^{a}	**.001**

L-PrE Group 1: Late-onset preeclampsia patient group, Control Group 2: Late-onset preeclampsia control group, E-PrE Group 3: Early-onset preeclampsia patient group, Control Group 4: Early-onset preeclampsia control group, Age: Maternal age, BMI: Body Mass Index, Gestational age: Gestational age at the time of recruitment, Syst TA: Systolic blood pressure, Diast TA: Diastolic blood pressure, n: Number, SD: Standard deviation, $p < .05$ indicates statistical significance; a,b,c: different letters symbolize the difference between the groups.

Table 2. The laboratory parameters of the groups					
Variables	**L-PrE group 1 (n=30)**	**Control group 2 (n=33)**	**E-PrE Group 3 (n=31)**	**Control group 4 (n=31)**	**p**
Hemoglobin (g/dL, mean±SD)	12.0±1.4[a]	10.9±1.3[b]	11.9±1.3[a]	11.4±1.2[ab]	**.004**
Hematocrit (%, mean±SD)	36±3[a]	33±3[c]	35±3[ab]	33±2[bc]	**.001**
Platelets ($x10^3/\mu L$, mean±SD)	240±84	235±82	200±83	236±51	.152
WBC (µL/mL, mean±SD)	10.9±2.46	10.5±2.6	10.4±3.0	9.9±2.8	.587
BUN (mg/dl, mean±SD)	8.9±3.0[ab]	7.3±2.5[bc]	10.4±3.9[a]	6.4±2.1[c]	**.001**
Creatinine (mg/dl, mean±SD)	0.56±0.11[b]	0.47±0.09[c]	0.66±0.17[a]	0.46±0.08[c]	**.001**
ALT (IU/l, mean±SD)	20±30	10±4	22±30	11±4	.054
AST (U/l, mean±SD)	22±17	16±3	31±35	14±3	**.005**
Proteinuria (mean±SD)	2.67±1.1[a]	0±0[b]	3.0±1.2[a]	0.0±0.2[b]	**.001**
s-ENG (ng/ml, mean±SD)	17.24±1.73[a]	18.49±2.01[ab]	22.64±12.98[b]	18.21±4.48[ab]	**.015**
MMP-14 (ng/ml, mean±SD)	2.83±0.31[a]	2.93±0.43[a]	3.82±0.94[b]	3.81±1.26[b]	**.001**

L-PrE Group 1: Late-onset preeclampsia patient group, Control Group 2: Late-onset preeclampsia control group, E-PrE Group 3: Early-onset preeclampsia patient group, Control Group 4: Early-onset preeclampsia control group, WBC: White blood cells, BUN: Blood urea nitrogen, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, Proteinuria: Spot urine proteinuria by dipstick test, s-ENG: Soluble Endoglin, MMP-14: Matrix metalloproteinase-14, n: Number, SD: Standard deviation, $p < .05$ indicates statistical significance; a,b,c: different letters symbolize the difference between the groups.

Table 3. The clinical characteristics and biomarkers levels of the preeclampsia groups.					
Variables		**Biomarkers**			
Group 1 and 3	**Subgroups**	**s-ENG (ng/ml, mean±SD)**	**p**	**MMP-14 (ng/ml, mean±SD)**	**p**
Nausea, vomiting and epigastric symptoms	None (n=117)	18.37±3.96	.162	3.30±0.92	.154

	Exist (n=8)	30.65±22.19		3.93±1.09	
Persistent cerebral symptoms	None (n=110)	19.00±7.19	.528	3.33±0.91	.573
	Exist (n=15)	20.26±7.30		3.47±1.21	
Preeclampsia	Mild (n=29)	18.61±5.88	.291	3.11±0.61	**.047**
	Severe (n=32)	21.24±12.06		3.54±1.00	
Small for gestational age	None (n=115)	18.87±6.56	.133	3.35±0.94	.969
	Exist (n=10)	22.43±12.38		3.33±1.04	
Group 1+3: Late-onset preeclampsia patient group and early-onset preeclampsia patient group, s-ENG: Soluble Endoglin, MMP-14: Matrix metalloproteinase-14, n: Number, SD: Standard deviation, p < .05 indicates statistical significance.					

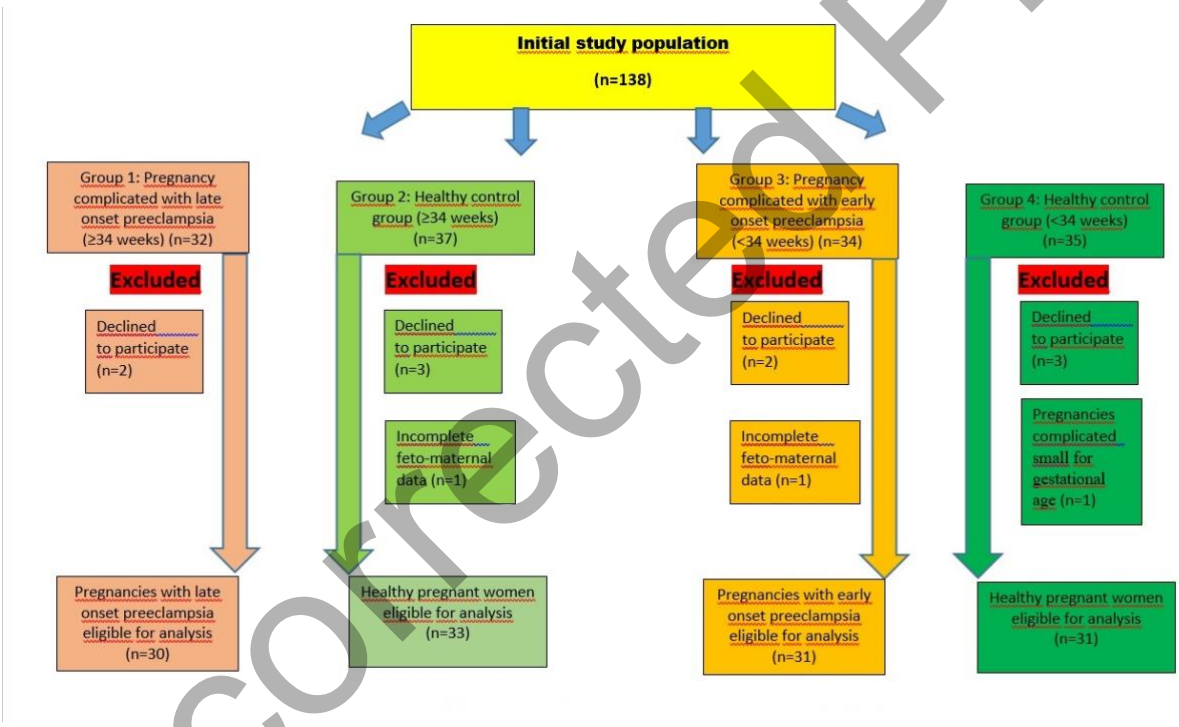

Figure 1. Flow chart of the pregnant women recruited in the study

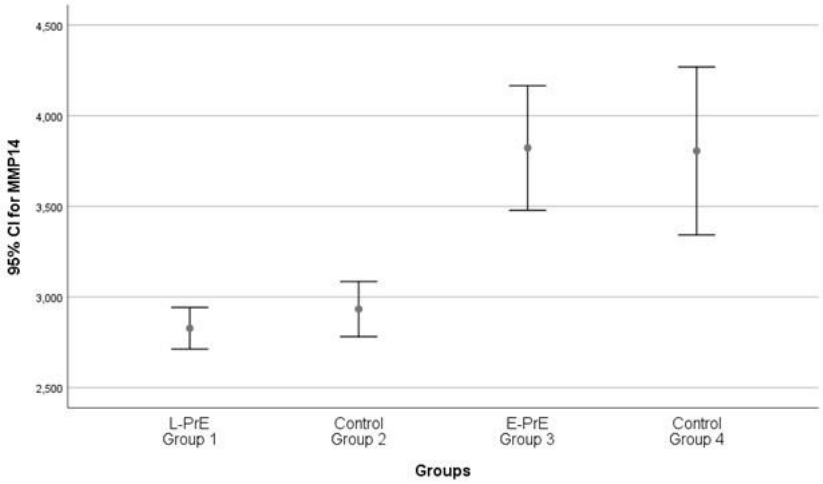

Figure 2. Box plot showing mean value of matrix metalloproteinase-14 in the groups

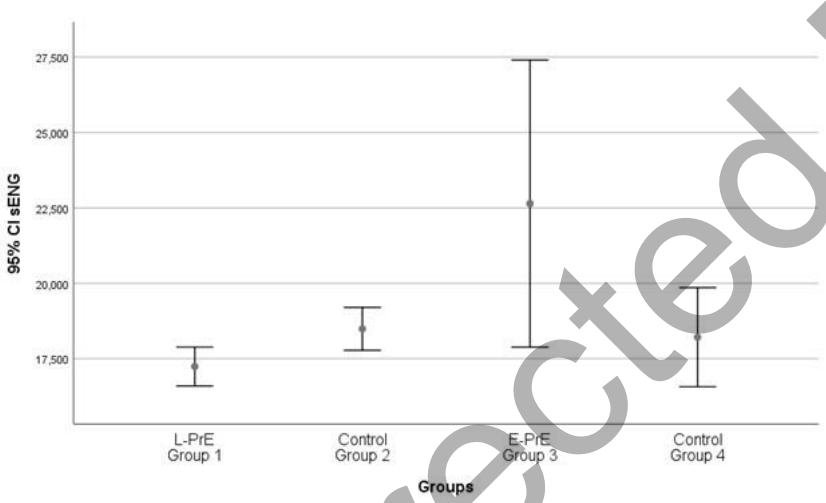

Figure 3. Box plot showing mean value of soluble endoglin in the groups