RESEARCH ARTICLE

# Promoter Methylation and Low Placental Expression of Metalloproteinase (MMP)-9, Human Leukocyte Antigen (HLA)-G, Vascular Endothelial Growth Factor (VEGF), and Highly Soluble Endoglin (sEng) as Risk Factors for Preeclampsia

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## Abstract

**BACKGROUND:** Dysregulated expressions of metalloproteinase (MMP)-9, human leucocyte antigen (HLA)-G, vascular endothelial growth factor (VEGF), and soluble endoglin (sEng) reflect impaired angiogenesis, immune tolerance, endothelial function, and trophoblast invasion that characterize abnormal placental development in preeclamptic (PE) pregnancies. However, the role of promoter methylation of these markers in linking the pathways to altered protein expression remains unclear. Hence, this study compared promoter methylation and placental expression of MMP-9, HLA-G, VEGF, and sEng between women with PE and normotensive pregnancies, and evaluate their diagnostic performance as potential biomarkers.

**METHODS:** This case–control study included 30 women with PE and 30 controls. Placental tissue samples were collected within 15 minutes postpartum. Placental promoter methylation was assessed using methylation-specific polymerase chain reaction (PCR), and protein expression was measured using enzyme-linked immunosorbent assay (ELISA). Group differences were analyzed, diagnostic accuracy was evaluated using receiver operating characteristic (ROC) curves, and associations were expressed as adjusted odds ratios (AOR).

**RESULTS:** Compared with controls, placentas from women with PE significantly showed higher methylation of *HLA-G* (58.9% *vs.* 37.3%) and *sEng* (6.7% *vs.* 4.1%), and lower methylation of *VEGF* (30.4% *vs.* 48.1%) and *MMP-9* (36.1% *vs.* 44.9%). Expression of MMP-9, HLA-G, and VEGF was significantly reduced, while sEng expression was increased in PE. Multivariate analysis identified *HLA-G* hypermethylation (AOR 5.36), *VEGF* hypomethylation (AOR 8.55), *sEng* methylation (AOR 4.57), low expression of MMP-9, HLA-G, and VEGF, and high sEng expression (AOR 4.77) as independent predictors of PE. sEng expression demonstrated the best discrimination (AUC 0.835), followed by *sEng* methylation (AUC 0.785) and *HLA-G* methylation (AUC 0.774).

**CONCLUSION:** PE is associated with distinct placental methylation–expression alterations, with sEng- and HLA-G–related markers showing the strongest diagnostic value.

**KEYWORDS:** preeclampsia, DNA methylation, angiogenesis, MMP-9, HLA-G, sEng, placenta, epigenetics

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## Introduction

Preeclampsia (PE) is a multisystem disorder of pregnancy that typically manifests in the second half of gestation, affecting around 5% of pregnancies worldwide and contributing significantly to maternal and perinatal morbidity and mortality.(1) Globally, PE is estimated to cause approximately 11%-14% maternal mortality annually. (2) It is one of the major contributors to maternal deaths, especially in low and middle-income countries. PE may also result in severe outcomes, such as restricted fetal growth and premature delivery.(3) Despite advances in obstetric care, the etiology and pathophysiology of PE remain incompletely understood. Among the several dominant risk factors, several theories have been developed regarding the pathomechanism of PE, *e.g.*, genetic predisposition, immunity, oxidative stress, inflammation, hypoxia, angiogenic imbalance, nutrition, and hormonal influences. (4–6) However, placental dysfunction, particularly inadequate trophoblast invasion and impaired remodeling of the spiral arteries, is recognized as a central pathological event in early-onset PE.(7)

A wealth of evidence shows that an imbalance between angiogenic and anti-angiogenic factors contributes significantly to the development of PE.(8) Critical pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and human leucocyte antigen (HLA)-G, play vital roles in placental vascularization and immune tolerance, respectively.(9,10) In contrast, soluble endoglin (sEng), an anti-angiogenic factor, has been identified as a potent inhibitor of TGF-β signaling, contributing to endothelial dysfunction in PE.(11) Another key player is matrix metalloproteinase (MMP)-9, an enzyme that regulates extracellular matrix remodeling and facilitates proper trophoblast invasion. The altered expression of these proteins has been consistently linked to abnormal placental development in preeclamptic pregnancies.(12,13)

Moreover, epigenetic modifications, particularly DNA methylation, have emerged as an essential regulator of gene expression in placental development and disease. DNA methylation predominantly occurs at cytosine residues within CpG islands in promoter regions and can lead to transcriptional silencing when hypermethylated or to gene activation when hypomethylated. In PE, unusual methylation patterns have been observed in genes regulating angiogenesis, immune tolerance, and extracellular matrix remodeling.(14,15) Given the multifactorial nature of PE, therefore this study was conducted to compare the promoter methylation and placental expression of MMP-9, HLA-G, VEGF, and sEng between women with PE and normotensive pregnancies, and to evaluate their diagnostic performance as potential biomarkers.

## Methods

### Study Design and Subjects Recruitment

This observational case–control study was conducted at Ngoerah Hospital, Denpasar, Bali, Indonesia, between January 2023 and December 2024. A total of 60 pregnant women were recruited, comprising 30 women with PE and 30 normotensive women with uncomplicated pregnancies (control group). The inclusion criteria included a maternal age between 18 and 45 years, singleton pregnancies, and gestational age >20 weeks. This study followed the American College of Obstetricians and Gynecologists' guideline for diagnosing PE. Thus, PE was diagnosed as having a systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg detected on two measurements with a minimum interval of 4 hours, after the pregnancy entered the age of 20 weeks, either accompanied or without proteinuria (≥300 mg in 24 hours or urine protein to creatinine ratio ≥0.3). The exclusion criteria included women with chronic hypertension, autoimmune disease, renal disease, multiple pregnancies, or infections.

The required sample size was determined a *priori* using a two-sided comparison of proportions, assuming an expected difference in methylation/expression prevalence between the PE and control groups based on previously published data.(14) Based on an α level of 0.05, power of 80%, and a 1:1 case-to-control ratio, the minimum required sample was 25 subjects per group. To account for possible drop-outs and laboratory failure, the authors increased the sample size to 30 women per group (total n=60). The study protocol was approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Udayana (Approval No. 2568/UN14.2.2.VII.14/LT/2024).

### Sample Collection

Placental tissue was collected immediately after delivery (within 15 minutes postpartum) to minimize ischemic changes. A ~1-cm³ fragment of villous tissue was excised from the maternal (basal) side, approximately 2–3 cm away from the cord insertion site and avoiding areas of infarction, calcification, or hemorrhage. The sample was rinsed in ice-cold PBS to remove blood. Then, it was blotted dry, placed in cryovials, snap-frozen, and stored at −80°C until analysis.

The storage duration before analysis was two months, and freeze–thaw cycles were avoided. All samples were processed in the Molecular Biology Laboratory, Universitas Udayana.

**DNA Methylation Analysis**

Genomic DNA (500–1000 ng) from each placental sample was subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Cat. No. D5005, Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The DNA was denatured at 98°C for 10 minutes and incubated at 64°C for 2.5 hours with the conversion reagent. Then, the DNA underwent desulfonation and column purification. Next, the converted DNA was eluted in 20 µL nuclease-free water and stored at −20°C until analysis. Conversion efficiency was verified using non-CpG cytosine control regions.

Methylation-specific polymerase chain reaction (PCR), or known as MSP, was performed using primer pairs designed to distinguish methylated and unmethylated CpG sites in the promoter regions of *MMP-9*, *HLA-G*, *VEGF*, and *sEng*. The primer sequences were presented in Table 1.

Each 25 µL PCR reaction contained 2 µL of bisulfite-converted DNA, 1× PCR buffer, 2.0 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of each primer, and 1 U Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Thermocycling conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. PCR products were resolved on 2% agarose gels, stained with GelRed, and visualized under UV illumination. Electrophoretic visualization yielded three possible methylation patterns: complete methylation (100%), indicated by the presence of only methylated bands; no methylation (0%), indicated by the presence of only unmethylated bands; and partial methylation (1–99%), indicated by the presence of both methylated and unmethylated bands.

All molecular analyses were performed under standardized laboratory protocols. Each methylation-specific PCR run included a fully methylated DNA positive control, an unmethylated negative control, and a no-template control to exclude contamination and ensure assay specificity. Although methylation-specific PCR primarily detects the presence or absence of promoter methylation, methylation density in this study was estimated using band-intensity analysis.

Semi-quantitative analysis of promoter DNA methylation was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) to measure the intensity of electrophoresis bands corresponding to methylated and unmethylated PCR products. For each sample, the percentage of DNA methylation was calculated by dividing the intensity of the methylated band by the sum of the intensities of the methylated and unmethylated bands, multiplied by 100. Accordingly, the reported methylation values represent relative semi-quantitative percentages for each gene promoter. Methylation levels of the *MMP-9*, *HLA-G*, *VEGF*, and *sEng* promoters were subsequently compared between groups using these ImageJ-derived measurements.

**Table 1. Primer sequences used for methylation-specific PC.**

| Gene | Methylation Status | Primer Sirection | Sequence (5'–3') |
|---|---|---|---|
| *MMP-9* | Methylated | Forward | GAAGTTCGAAATTAGTTTGGTTAAC |
| | | Reverse | TCCCGAATAACTAATATTATAAACGTA |
| | Unmethylated | Forward | AGTTTGAAATTAGTTTGGTTAATGT |
| | | Reverse | CCTCCCAAATAACTAATATTATAAACATA |
| *HLA-G* | Methylated | Forward | TTAGGGAGATATTGAGATAGAACGT |
| | | Reverse | AATAAAAATAAAAACTAAAACCGCC |
| | Unmethylated | Forward | TTAGGGAGATATTGAGATAGAATGT |
| | | Reverse | CCTAATAAAAATAAAAACTAAAACCACC |
| *VEGF* | Methylated | Forward | CAGAAGGAGGAGGGCAGAAT |
| | | Reverse | CACACAGGATGGCTTGAAGA |
| | Unmethylated | Forward | GGAGGCCAGGCATAGAGAGA |
| | | Reverse | TTTCCCTTTTCTCGAAGTGA |
| *sEng* | Methylated | Forward | CCACTAGCCAGGTCTCGAAG |
| | | Reverse | GATGCAGGAAGACACTGCTG |
| | Unmethylated | Forward | GATGGAGCAGGGATGTGTG |
| | | Reverse | AAACCAACCAAAACCAACCA |

**Protein Expressions Analysis**

Approximately 50–100 mg of placental tissue was homogenized in ice-cold lysis buffer containing protease inhibitors and centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was collected and stored at −80°C until analysis. Then, concentrations of MMP-9, VEGF, and sEng were measured using sandwich ELISA kits (Human MMP-9 ELISA Kit, Cat. No. RAB0372, Sigma-Aldrich, St. Louis, MO USA; Human VEGF ELISA Kit, Cat. No. RAB0508, Sigma-Aldrich; Human Endoglin ELISA Kit, Cat. No. RAB0171, Sigma-Aldrich, respectively). For HLA-G, a validated research-use ELISA kit (HLA-G ELISA Kit, Cat. No. MBS267094, MyBioSource, San Diego, CA, USA) was used. Next, the samples were analyzed in duplicate and diluted to fall within the linear portion of the standard curves (typical working dilutions: 1:10–1:50 for VEGF and MMP-9, and 1:50–1:100 for HLA-G and sEng). Then, the standard curves for each assay were generated using seven serial dilutions, and the concentrations were calculated using a four-parameter logistic regression model. The Bradford assay was then performed to determine the total protein concentration, and target protein levels were normalized to total protein content. Assay precision was monitored throughout the procedure; intra-assay coefficients of variation were maintained below 8%, and inter-assay coefficients below 10%. Plates exceeding these thresholds were repeated.

For ELISA assays, each plate included blank wells, high- and low-concentration controls, and a full standard curve. All samples were analyzed in duplicate. Laboratory personnel performing ELISA assays were blinded to the patients' clinical status (PE *vs.* control) until all measurements were completed. Reproducibility was assessed by repeating 10% of randomly selected samples, which demonstrated high concordance across runs.

**Statistical Analysis**

To analyze the data, SPSS 26 (IBM Corporation, Armonk, NY, USA) was used in this study. The continuous data in this study are reported as means±standard deviations (SD) or as medians. The categorical variables are described as frequencies and proportions. Group differences were analyzed based on the type and distributions of the data. Additionally, the receiver operating characteristic (ROC) curves, and adjusted odds ratios (AOR) values were also analyzed to evaluate diagnostic performance. The odd ration (OR) and 95% CI were also calculated to assess the risk association. A $p<0.05$ is considered statistically significant.

## Results

**Subjects Characteristics**

This study involved 60 pregnant women, divided into two groups: 30 with PE (case group) and 30 without PE (control group). The subjects' characteristics, including age, parity, and body mass index (BMI) were compared between the two groups, and in presented in Table 2. The normality test

**Table 2. Subjects' characteristics and their correlation with PE Incidence.**

| Characteristics | PE (n=30) | Non-PE (n=30) | OR | 95% CI | *p*-value |
|---|---|---|---|---|---|
| Age (years), Mean±SD | 29.57±7.7 | 27.40±6.54 | | | 0.214[a] |
| Age (years), n (%) | | | | | |
| ≤20 | 5 (16.7) | 2 (6,7) | 7.00 | 1.38-35.47 | 0.021[b]* |
| 21-35 | 17 (56.7) | 26 (86.7) | | | |
| >35 | 8 (26.7) | 2 (6,7) | | | |
| Parity, Mean±SD | 1.73±1.28 | 1.07±0.91 | | | 0.089[a] |
| Parity, n (%) | | | | | |
| Nullipara | 4 (13.3) | 9 (30.0) | 0.36 | 0.09-1.33 | 0.117[b] |
| Parity ≥1 | 26 (86.7) | 21 (70.0) | | | |
| BMI (kg/m$^2$), Mean±SD | 24.66±1.93 | 22.05 ±1.58 | | | <0.001[a]* |
| BMI (kg/m$^2$), n (%) | | | | | |
| <18.5 | 0 (0) | 1 (3,3) | 9.33 | 2.84-30.60 | <0.001[b]* |
| 18.5-22.9 | 9 (30.0) | 23 (76.7) | | | |
| 23-24.9 | 7 (23.3) | 5 (16.7) | | | |
| ≥25 | 14 (46.7) | 1 (3,3) | | | |

*Significant if $p<0.05$; [a]Tested with Independent T-test, [b]Tested with Chi-square test.

results showed that age, parity, and BMI data were normally distributed ($p$>0.05).

**_HLA-G_ and _sEng_ Promoter Methylation as well as sEng and VEGF Expressions Showed Strongest Discrimination between Groups**

The methylation levels of the *MMP-9*, *HLA-G*, *VEGF*, and *sEng* gene promoters were compared between the groups. The ROC curve analysis showed that all evaluated methylation and expression markers exhibited measurable discriminatory ability for PE. However, the magnitude of diagnostic performance varied across biomarkers. Among the promoter methylation markers, *HLA-G* and *sEng* demonstrated the strongest discrimination (AUCs of 0.774 and 0.785, respectively), whereas *MMP-9* and *VEGF* methylation showed lower diagnostic utility. Regarding protein expression, sEng provided the highest accuracy (AUC 0.835), followed by VEGF (AUC 0.734) (Figure 1, Figure 2, Table 3).

**Bivariate Analysis of PE Risk Factors**

The bivariate analysis showed that both epigenetic and expression abnormalities in MMP-9, HLA-G, VEGF, and sEng were significantly associated with PE. Low *MMP-9* methylation and reduced MMP-9 expression were each associated with a ~5-fold increased risk of PE, consistent with impaired trophoblast invasion. Meanwhile, *HLA-G* hypermethylation and low HLA-G expression indicate strong associations with disease, conferring a 6–16-fold higher risk and reflecting disruption of maternal immune tolerance. The *VEGF* hypomethylation and low VEGF expression were also significant risk factors, which align with defective angiogenesis. Both *sEng* promoter methylation and elevated circulating sEng also significantly increased PE risk (Table 4).

**Multivariate Analysis of Overall PE Risk Factors**

The multivariate analysis included all known risk and epigenetic factors linked to PE examined in this study (Table 5). The multivariate analysis revealed that the significant risk factors for PE in this study were obesity (BMI >25 kg/m$^2$), DNA hypermethylation of the *HLA-G* gene (>51.15%), low HLA-G expression (<194.02 µg/mL), DNA hypomethylation of the *VEGF* gene (<37%), low VEGF expression (≤4.62 ng/mL), methylated DNA of the *sEng* gene, high sEng expression (>22.875 µg/mL), low DNA methylation of the *MMP-9* gene (≤49%), and low MMP-9 expression (≤6.81 µg/mL). In this study, older maternal age and nulliparity were not significantly associated with the PE risk.

Figure 3 illustrated the relationships between the methylation levels of the *sEng*, *VEGF*, *MMP-9*, and *HLA-G* genes, their impact on protein expression, and the incidence of PE. In this model, increased methylation of the *sEng* gene leads to increased protein expression, ultimately increasing the risk of PE. Conversely, decreased methylation of the

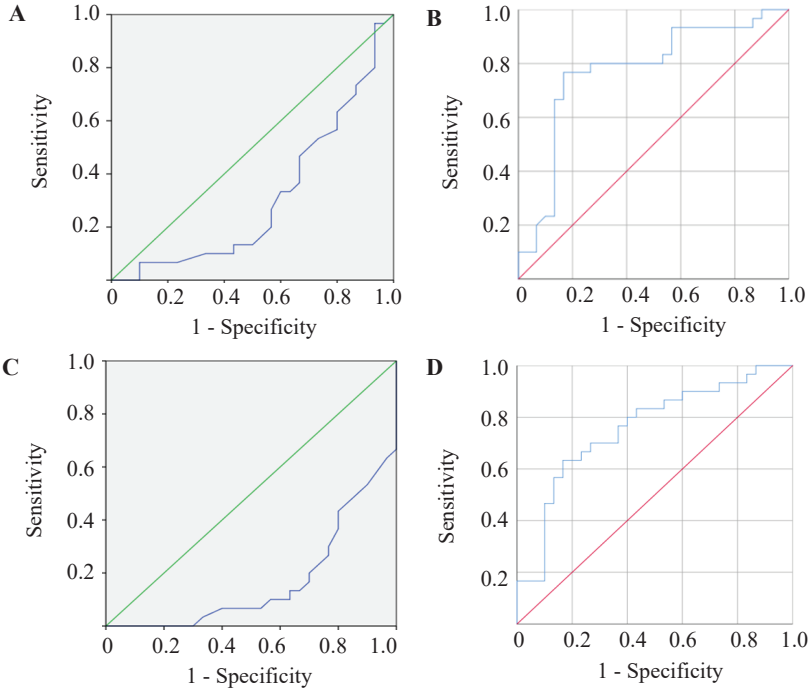


**Figure 1. ROC curves of the gene promoter methylations levels in predicting PE.** A: *MMP-9*; B: *HLA-G*; C: *VEGF*; D) *sEng*.

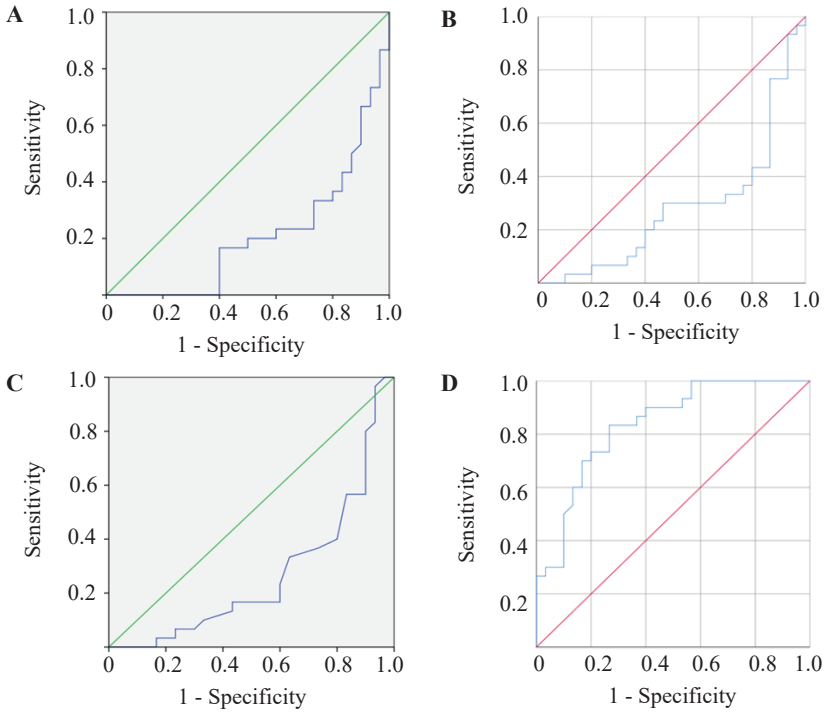


**Figure 2. ROC curves of the protein expression levels in predicting PE.** A: MMP-9; B: HLA-G; C: VEGF; D) sEng.

*VEGF* and *MMP-9* genes results in reduced expression, which is also associated with an increased risk of PE. Meanwhile, increased methylation of the *HLA-G* gene decreases its expression, increasing the likelihood of PE.

In this path analysis, the indirect effect of genetic methylation on the incidence of PE is calculated by multiplying the path coefficient for the relationship between gene methylation and its protein expression by the path coefficient for protein expression on the incidence of PE. For example, the indirect effect of *VEGF* methylation is calculated by multiplying the coefficient for *VEGF* methylation and VEGF expression (0.367) by the coefficient for VEGF expression and PE (-0.370), yielding an indirect effect value of -0.13579. The same calculation method was applied to all other pathways: *sEng* methylation had an indirect effect of 0.339 × 0.390 = 0.13220; *MMP-9* methylation of 0.400 × (−0.185) = −0.0740; and *HLA-G* methylation of (−0.143) × (−0.310) = 0.04433. These indirect effect values were then compared to determine which epigenetic variable had the most significant influence on the occurrence of PE in the path analysis model.

Furthermore, the path analysis showed that among the genetic methylation factors studied, decreased methylation of the *VEGF* gene promoter has the most significant indirect effect on the incidence of PE, with an indirect effect of -0.1358. Decreased *VEGF* methylation contributes to decreased VEGF expression in the placenta, ultimately increasing the risk of PE. Meanwhile, increased methylation of the *sEng* gene promoter was found to significantly contribute to PE incidence, with an indirect effect of +0.1322,

**Table 3. ROC curves analysis of MMP-9, HLA-G, VEGF, and sEng promoter methylation and expression levels in predicting PE.**

| Parameter | PE (n=30) | Non-PE (n=30) | *p*-value | AUC | Cut-off | Sn | Sp |
|---|---|---|---|---|---|---|---|
| *MMP-9* methylation (%) | 36.13±10.90 | 44.86±13.97 | 0.012[a] | 0.311 | 49.0 | 13% | 53% |
| *HLA-G* methylation (%) | 58.93±20.07 | 37.32±22.98 | <0.001[a] | 0.774 | 51.1 | 76.7% | 83.3% |
| *VEGF* methylation (%) | 30.36±11.45 | 48.10±13.48 | <0.001[a] | 0.279 | 37.0 | 37% | 37% |
| *sEng* methylation (%) | 6.66±3.90 | 4.12±2.18 | 0.001[a] | 0.785 | 4.31 | 70% | 70% |
| MMP-9 expression (µg/mL) | 5.07±2.09 | 7.78±2.42 | <0.001[a] | 0.209 | 6.81 | 23% | 40% |
| HLA-G expression (µg/mL) | 215.88±100.36 | 468.57±614.24 | 0.004[a] | 0.282 | 194.02 | 63.33% | 80% |
| VEGF expression (ng/mL) | 5.49±6.77 | 27.05±57.05 | 0.002[a] | 0.734 | 4.62 | 73% | 63% |
| sEng expression (µg/mL) | 34.08±14.72 | 16.99±10.07 | <0.001[a] | 0.835 | 22.87 | 73% | 73% |

[a]Tested with Mann-Whitney. AUC: area under the curve, Sn: sensitivity, Sp: specificity.

**Table 4. Bivariate analysis between MMP-9, HLA-G, VEGF, sEng gene promoter methylation and protein expression as risk factors of PE.**

| Variables | n (%) | | OR | 95% CI | *p* -value |
|---|---|---|---|---|---|
| | PE (n = 30) | Non-PE (n = 30) | | | |
| *MMP-9* Methylation | | | | | |
| Low (≤49%) | 26 (86.7%) | 16 (53.3%) | 5.68 | 1.59 – 20.33 | 0.005[a] |
| High (>49%) | 4(13.3%) | 14 (46.7%) | | | |
| MMP-9 Expression | | | | | |
| Low (≤6.81 µg/mL) | 23 (76.7%) | 12 (40.0%) | 4.92 | 1.61 – 15.07 | 0.004[a] |
| High (>6.81 µg/mL) | 7 (23.3%) | 18 (60.0%) | | | |
| *HLA-G* Methylation | | | | | |
| Hypermethylation (>51.15%) | 23 (76.7%) | 5 (16.7%) | 16.429 | 4.569-59.073 | <0.001[a] |
| Normal Methylation (≤51.15%) | 7 (23.3%) | 25 (83.3%) | | | |
| HLA-G Expression | | | | | |
| Low (<194.02 µg/mL) | 19 (63.3%) | 6 (20.0%) | 6.909 | 2.160-22.098 | 0.001[a] |
| Normal (≥194.02 µg/mL) | 11 (36.7%) | 24 (80.0%) | | | |
| *VEGF* Methylation | | | | | |
| Hypomethylation (<37%) | 22 (73.3%) | 7 (23.3%) | 9.0 | 2.80 – 29.13 | <0.001[a] |
| Normal methylation (≥37%) | 8 (26.7%) | 23 (76.7%) | | | |
| VEGF Expression | | | | | |
| Low (≤4.62 ng/mL) | 19 (63.3%) | 8 (26.7%) | 4.75 | 1.58 – 14.2 | 0.004[a] |
| High (>4.62 ng/mL) | 11 (36.7%) | 22 (73.3%) | | | |
| *sEng* Methylation | | | | | |
| Methylated | 21 (70.0%) | 9 (30.0%) | 5.44 | 1.80-16.43 | 0.002[a] |
| Unmethylated | 9 (30.0%) | 21 (70.0%) | | | |
| sEng Expression | | | | | |
| High sEng (>22.875 µg/mL) | 22 (73.3%) | 8 (26.7%) | 7.56 | 2.41-23.75 | <0.001[a] |
| Low sEng (≤22.875 µg/mL) | 8 (26.7%) | 22 (73.3%) | | | |

[a]Tested with Chi-square.

via increased placental sEng expression, which plays a role in endothelial dysfunction. *MMP-9* hypomethylation and *HLA-G* hypermethylation also contribute to the incidence of PE, but their effects are smaller than *VEGF* and *sEng*.

## Discussion

This results of study demonstrates that PE is associated with epigenetic and expression disturbances in placental genes that regulate trophoblast invasion (*MMP-9*), immune tolerance (*HLA-G*), angiogenesis (*VEGF*), and endothelial function (*sEng*). The findings support an integrated placental dysfunction model in which unusual promoter methylation alters gene expression, contributing to impaired spiral artery remodeling, immune dysregulation, and angiogenic imbalance, which are hallmark features of PE.(11,12)

Within this integrated framework, MMP-9 dysregulation appears to contribute to impaired trophoblast invasion. Although promoter hypomethylation is generally associated with transcriptional activation, our findings showed that hypomethylation was accompanied by reduced placental MMP-9 expression, suggesting altered epigenetic control rather than linear methylation–expression regulation.(13–15) Similar complexity has been reported in previous studies, where imbalances in metalloproteinase systems, including MMP-2/tissue inhibitor of metalloproteinases (TIMP)-2 and related enzymes, disrupt extracellular matrix remodeling and placental implantation.(16) Tet Methylcytosine Dioxygenase (TET)2-mediated demethylation of the *MMP-9* promoter modulates trophoblast migration, underscoring the role of dynamic epigenetic regulation in placental development. (17) Reduced MMP-9 activity has also been associated with impaired vasodilation, limited uterine expansion, and hypertensive complications of pregnancy, including PE.(12) Moreover, some previous clinical studies have reported that various MMP-9 levels across gestational stages affect

**Table 5. Multivariate analysis of PE risk factors.**

| Variables | AOR | 95%CI | *p*-value |
|---|---|---|---|
| Advanced maternal age (>35 years) | 1.972 | 0.442-8.796 | 0.374 |
| Obesity (BMI >25 kg/m$^2$) | 8.099 | 1.612-40.685 | 0.011* |
| Nullipara | 0.005 | 0.000-2.66 | 0.099 |
| DNA hypermethylation of *HLA-G* gene (>51.15%) | 5.364 | 1.206-23.856 | 0.027* |
| Low HLA-G expression (<194.02 µg/mL) | 4.520 | 1.103-18.516 | 0.036* |
| DNA hypomethylation of the *VEGF* gene (<37%) | 8.549 | 1.748-41.809 | 0.008* |
| Low VEGF expression (≤4.62 ng/mL) | 4.778 | 1.079-21.158 | 0.039* |
| Methylated *sEng* gene DNA | 4.567 | 1.040-20.059 | 0.044* |
| High sEng expression (>22.875 µg/mL) | 4.769 | 1.221-18.635 | 0.025* |
| DNA hypomethylation of *MMP-9* gene (≤49%) | 7.162 | 1.291-39.719 | 0.024* |
| Low MMP-9 expression (≤6.81 µg/mL) | 4.477 | 1.092-18.354 | 0.037* |

*Significant if $p<0.05$.

disease severity, further supporting the complexity of MMP-9 regulation in PE.(13,18,19)

This study also found that *HLA-G* hypermethylation, associated with reduced placental expression, was a strong, independent risk factor. These findings are consistent with previous studies that found increased methylation in key *HLA-G* promoter regions (–188, –68, –65) in preeclamptic pregnancies.(20) Reduced HLA-G expression compromises maternal–fetal immune tolerance by weakening inhibitory signaling through immunoglobulin-like transcripts, leading to heightened activation of decidual immune cells and impaired spiral artery remodeling.(20,21) The resulting inflammatory environment, characterized by increased pro-inflammatory cytokines, further exacerbates endothelial dysfunction and placental ischemia. Although existing evidence supports these mechanisms, limited population-based studies on *HLA-G* methylation warrant cautious interpretation and highlight the need for larger confirmatory studies.(20,21)

In terms of angiogenic regulation, *VEGF* promoter hypomethylation, accompanied by reduced VEGF expression, was significantly associated with increased PE risk. Similar findings have been reported in a previous studies, where lower *VEGF* promoter methylation in preeclamptic placentas.(22) Decreased VEGF expression has been consistently reported across multiple populations, including studies from Iraq, Korea, and Uganda, although diagnostic performance varies by gestational age and disease severity.(23–25) Conversely, other studies have reported elevated VEGF levels in PE, a discrepancy likely attributable to methodological differences in VEGF measurement and the influence of platelet-derived VEGF.(26–28) Notably,

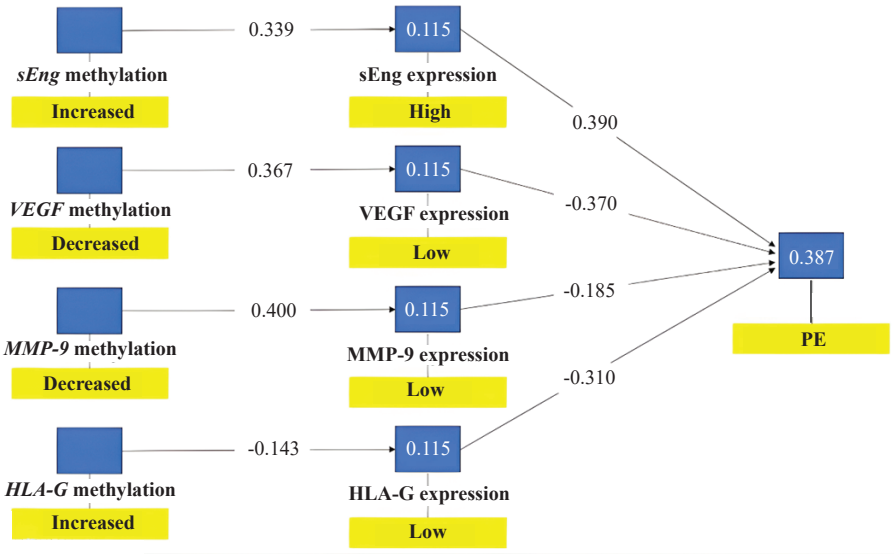


**Figure 3. Path analysis results of PE risk factors.**

sandwich-type ELISA primarily detects free VEGF, whereas bound VEGF complexes increase during pregnancy due to elevated VEGF-binding proteins.(29) Additionally, increased circulating soluble Fms-like tyrosine kinase 1 (sFlt-1) in PE neutralizes VEGF bioavailability, further contributing to endothelial dysfunction despite apparent promoter hypomethylation.(23,25,30)

Among all of the markers evaluated, sEng demonstrated the most consistent epigenetic, expression, and diagnostic association with PE. Increased *sEng* promoter methylation and elevated placental sEng expression were significantly associated with disease risk, consistent with previous studies reporting higher *sEng* methylation and circulating sEng levels in preeclamptic pregnancies.(31–33) As a key modulator of TGF-β signaling, excessive sEng disrupts endothelial homeostasis and vascular remodeling, promoting systemic endothelial dysfunction, a central feature of PE.(32,33) Elevated sEng also acts synergistically with other anti-angiogenic factors, including sFlt-1, amplifying vascular resistance and impairing uteroplacental perfusion.(34) Longitudinal studies further support sEng as a predictive biomarker, with rising levels detectable weeks to months before clinical disease onset.

Nevertheless, this study has limitations, including its relatively small sample size and single-center design, which may limit generalizability and precision of risk estimates. The semi-quantitative nature of MSP also restricts absolute methylation quantification. However, this study's strengths include the integrated epigenetic–expression approach, standardized sample handling, and comprehensive multivariate and diagnostic analyses conducted under strict quality control. Clinically, these findings suggest that combined epigenetic–protein signatures, particularly involving HLA-G and sEng, may offer greater diagnostic and risk-stratification value than individual markers alone. However, translation into clinical screening requires validation in larger, multi-center cohorts and longitudinal assessment earlier in gestation.

## Conclusion

This study demonstrates that alterations in promoter methylation and placental expression of MMP-9, HLA-G, VEGF, and sEng are significantly associated with PE. These alterations reflect coordinated disturbances in pathways related to placental invasion, immune regulation, angiogenesis, and endothelial function. Although causality cannot be inferred, the consistent associations observed support their relevance to PE pathophysiology and warrant further investigation in larger and longitudinal studies.

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## Authors Contribution

AANJK. conceived and designed the study, served as the principal investigator, and was responsible for data collection and data analysis. IMD, KM, GPB, CGAAN, CS, and NNWTM contributed to subject recruitment, data acquisition, and provided methodological and clinical support during the study. All authors contributed to data interpretation, critically revised the manuscript for important intellectual content, and approved the final version of the manuscript.

## Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript. The funding agency had no role in designing the study, collecting and analyzing data, making publication decisions, or preparing the manuscript.

## References

1. Vera-Ponce VJ, Loayza-Castro JA, Ballena-Caicedo J, Valladolid-Sandoval LAM, Zuzunaga-Montoya FE, Gutierrez De Carrillo CI. Global prevalence of preeclampsia, eclampsia, and HELLP syndrome: A systematic review and meta-analysis. Front Reprod Heal. 2025; 7: 1706009. doi:10.3389/frph.2025.1706009
2. Melesse MF, Aynalem GL, Badi MB, Aynalem BY. Maternal outcomes of severe preeclampsia and eclampsia and associated factors among women admitted at referral hospitals of amhara regional state, institutional-based cross-sectional study, North-West Ethiopia. Front Glob women's Heal. 2025; 6: 1555778. doi: 10.3389/fgwh.2025.1555778
3. Nelson N, Yusrawati Y, Serudji J, Jamsari J, Amir A AA. Elevation of miR-210 expression and mean arterial pressure as early-onset pre-eclampsia biomarkers, while wlevation of matrix metalloproteinase-2 as late-onset pre-eclampsia biomarker. Indones Biomed J. 2025; 17(2): 180-7.

4. Wantania J, Bakri S, Pandelaki K, Chalid M. Altered level of soluble fms-like tyrosine kinase 1 (sFlt1) and hypoxia inducible factor-1α (HIF-1α) in normotensive pregnancy and preeclampsia. Indones Biomed J. 2014; 5(2):121-8.
5. Putri RWR, Prasmusinto D, Wibowo N, Irwinda R, Purwosunu Y, Saroyo YB. Higher trace elements and lower fatty acids levels in erythrocytes as predictors of preeclampsia. Indones Biomed J. 2024; 16(6): 560-71.
6. Hudila Rifa Karmia Y, Jamsari, Hardisman. miR-200a as potential early-onset biomarker, while high nitric oxide as potential late-onset biomarker in preeclampsia. Indones Biomed J. 2025; 17(3): 273-8.
7. Jena MK, Sharma NR, Petitt M, Maulik D, Nayak NR. Pathogenesis of preeclampsia and therapeutic approaches targeting the placenta. Biomolecules. 2020; 10(6): 953. doi: 10.3390/biom10060953.
8. Rana S, Burke SD, Karumanchi SA. Imbalances in circulating angiogenic factors in the pathophysiology of preeclampsia and related disorders. Am J Obstet Gynecol. 2022; 226(2S): S1019-S1034. doi: 10.1016/j.ajog.2020.10.022.
9. Chiang YT, Seow KM, Chen KH. The pathophysiological, genetic, and hormonal changes in preeclampsia: A systematic review of the molecular mechanisms. Int J Mol Sci. 2024; 25(8): 4532. doi: 10.3390/ijms25084532.
10. Zouganeli I, Moustakli E, Potiris A, Christodoulaki C, Arkoulis I, Kathopoulis N, *et al.* Genetic variations in vascular endothelial growth factor and their impact on preeclampsia: Insights into risk, severity, and pregnancy outcomes. Curr Issues Mol Biol. 2025; 47(3): 199. https://doi.org/10.3390/cimb47030199.
11. Leaños-Miranda A, Navarro-Romero CS, Sillas-Pardo LJ, Ramírez-Valenzuela KL, Isordia-Salas I, Jiménez-Trejo LM. Soluble endoglin as a marker for preeclampsia, its severity, and the occurrence of adverse outcomes. Hypertens. 2019; 74(4): 991-7.
12. Chen J, Khalil RA. Matrix metalloproteinases in normal pregnancy and preeclampsia. Prog Mol Biol Transl Sci. 2017; 148: 87-165.
13. Timokhina E, Strizhakov A, Ibragimova S, Gitel E, Ignatko I, Belousova V, *et al.* Matrix metalloproteinases MMP-2 and MMP-9 occupy a new role in severe preeclampsia. J Pregnancy. 2020; 2020: 8369645. doi:10.1155/2020/8369645.
14. Ashraf UM, Hall DL, Rawls AZ, Alexander BT. Epigenetic processes during preeclampsia and effects on fetal development and chronic health. Clin Sci. 2021; 135(19): 2307-27.
15. Nilsson EE, Winchester P, Proctor C, Beck D, Skinner MK. Epigenetic biomarker for preeclampsia-associated preterm birth and potential preventative medicine. Environ epigenetics. 2024; 10(1): dvae022. doi: 10.1093/eep/dvae022.
16. Rahat B, Sharma R, Bagga R, Hamid A, Kaur J. Imbalance between matrix metalloproteinases and their tissue inhibitors in preeclampsia and gestational trophoblastic diseases. Reproduction. 2016; 152(1): 11-22.
17. Li X, Wu C, Shen Y, Wang K, Tang L, Zhou M, *et al.* Ten-eleven translocation 2 demethylates the MMP9 promoter, and its down-regulation in preeclampsia impairs trophoblast migration and invasion. J Biol Chem. 2018; 293(26): 10059-70.
18. Meng H, Qi M, Liu Y, Xu L, Li Q. Research on correlation between MMP-9 and early-onset preeclampsia. Int J Clin Exp Med. 2016; 9(9): 17442-8.
19. Zhou J, Yang S, Xu X, Xu X, Wang X, Ye A, *et al.* Screening of placenta accreta spectrum disorder using maternal serum biomarkers and clinical indicators: A case-control study. BMC Pregnancy Childbirth. 2023; 23(1): 508. doi: 10.1186/s12884-023-05784-2.
20. Tang Y, Liu H, Li H, Peng T, Gu W, Li X. Hypermethylation of the HLA-G promoter is associated with preeclampsia. Mol Hum Reprod. 2015; 21(9): 736-44.
21. Maghsudlu S, Ghazanfari Hashemi M, Talebi V, Vahabi E, Sharifpour S, *et al.* Association between serum HLA-G levels in the first trimester of pregnancy and the onset of preeclampsia: A systematic review and meta-analysis study. Int J Fertil Steril. 2023; 17(4): 231-5.
22. Sundrani DP, Reddy US, Joshi AA, Mehendale SS, Chavan-Gautam PM, Hardikar AA, *et al.* Differential placental methylation and expression of VEGF, FLT-1 and KDR genes in human term and preterm preeclampsia. Clin Epigenetics. 2013; 5(1): 6. doi: 10.1186/1868-7083-5-6.
23. Al-Ghazali B, Khadim M, Hamza S, Al-Haddad HS, Jumaah AS, Hadi N. Identification of vascular endothelial growth factor in preeclampsia in Iraqi women. J Med Life. 2022; 15(10): 1252-6.
24. Lee ES, Oh MJ, Jung JW, Lim JE, Seol HJ, Lee KJ, *et al.* The levels of circulating vascular endothelial growth factor and soluble Flt-1 in pregnancies complicated by preeclampsia. J Korean Med Sci. 2007; 22(1): 94-8.
25. Nabweyambo S, Kanyerezi S, Petterson JH, Katabazi FA, Ssekagiri A, Mwesigwa S, *et al.* No association of a vascular endothelial growth factor A ( VEGFA ) gene polymorphism with pre - eclampsia among pregnant women in Uganda. BMC Genomics. 2023; 24(1): 132. doi: 10.1186/s12864-023-09213-8.
26. Tandon V, Hiwale S, Amle D, Nagaria T, Patra PK. Assessment of serum vascular endothelial growth factor levels in pregnancy-induced hypertension patients. J Pregnancy. 2017; 2017: 3179670. doi: 10.1155/2017/3179670.
27. Baker PN, Krasnow J, Roberts JM, Yeo KT. Elevated serum levels of vascular endothelial growth factor in patients with preeclampsia. Obstet Gynecol. 1995; 86(5): 815-21.
28. Puspasari A, Enis RN, Herlambang. Genetic variant of vascular endothelial growth factor (VEGF)-A rs699947 is associated with preeclampsia. Mol Cell Biomed Sci. 2022; 6(2): 70-6.
29. Melincovici CS, Boşca AB, Şuşman S, Mărginean M, Mihu C, Istrate M, *et al.* Vascular endothelial growth factor (VEGF) - key factor in normal and pathological angiogenesis. Rom J Morphol Embryol. 2018; 59(2): 455-67.
30. Serudji J, Basyir V, Fadhillah T. Differentiating maternal angiogenesis factors between early and late onset preeclampsia: Higher sflt-1 in early onset preeclampsia, lower PlGF and higher sflt-1/PlGF ratio in late onset preeclampsia. Mol Cell Biomed Sci. 2024; 8(3): 154-8.
31. Kusuma AANJ, Darmayasa IM, Surya IGNHW, Wiradnyana AAGP, Winata IGS, Nugraha CGAA, *et al.* Methylation of endoglin gene promotor region and high soluble endoglin expression in placenta as risk factors for preeclampsia. Intisari Sains Medis. 2025; 16(2): 633-8.
32. Margioula-Siarkou G, Margioula-Siarkou C, Petousis S, Margaritis K, Vavoulidis E, Gullo G, *et al.* The role of endoglin and its soluble form in pathogenesis of preeclampsia. Mol Cell Biochem. 2021; 477(2): 479-91.
33. Nikuei P, Rajaei M, Malekzadeh K, Nejatizadeh A, Mohseni F, AtashAbParvar A. Accuracy of soluble endoglin for diagnosis of preeclampsia and its severity. Iran Biomed J. 2017; 21(5): 312-30.
34. Liu Z, Afink GB, Dijke PT. Soluble fms-like tyrosine kinase 1 and soluble endoglin are elevated circulating anti-angiogenic factors in pre-eclampsia. Pregnancy Hypertens. 2012; 2(4): 358-67.