RESEARCH ARTICLE

Hypomethylation of the Soluble Fms-like Tyrosine Kinase 1 (*sFlt-1*) Gene Promoter Region and Elevated sFlt-1 Placental Expression as Risk Factors for Preeclampsia

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Abstract

BACKGROUND: Preeclampsia significantly contributes to maternal and fetal morbidity and mortality worldwide, marked by an imbalance of angiogenic factors, particularly increased soluble Fms-like tyrosine kinase-1 (sFlt-1), leading to endothelial dysfunction. Epigenetic regulation, including DNA methylation of the *sFlt-1* promoter, has been suggested to influence sFlt-1 expression, but the data in Indonesian population are limited. This study was perfmed to determine whether hypomethylation of the *sFlt-1* promoter and elevated placental sFlt-1 expression are associated with increased risk of preeclampsia.

METHODS: A case-control study was conducted involving 30 women with preeclampsia and 30 normotensive pregnant women. Subjects were selected based on eligibility criteria that included singleton pregnancy and gestational age of ≥37 weeks. DNA methylation of the *sFlt-1* promoter was assessed using methylation-specific polymerase chain reaction (PCR), and sFlt-1 expression was measured by enzyme-linked immunosorbent assay (ELISA). Statistical analyses, including Mann-Whitney U, Chi-square tests, Receiver-operating characteristic (ROC) curve analysis, and multivariate logistic regression, were performed to evaluate the relationship between methylation levels, gene expression, and preeclampsia risk.

RESULTS: The preeclampsia group had significantly lower methylation levels of *sFlt-1* promoter and higher placental sFlt-1 expression (both $p<0.001$). Hypomethylation of *sFlt-1* promoter (adjusted odd ratio (AOR): 21.18; 95% CI: 2.49–179.72; $p=0.005$), high sFlt-1 expression (AOR: 12.55; 95% CI: 1.95–80.83; $p=0.008$), and obesity (AOR: 11.15; 95% CI: 2.01–61.78; $p=0.006$) were identified as independent risk factors for preeclampsia.

CONCLUSION: Hypomethylation of *sFlt-1* promoter and elevated placental sFlt-1 expression are significant independent risk factors for preeclampsia. These findings suggest that hypomethylation of *sFlt-1* promoter and elevated placental sFlt-1 expression may serve as potential epigenetic biomarkers for early detection and targeted intervention in preeclampsia.

KEYWORDS: preeclampsia, sFlt-1, gene expression, hypomethylation, placenta, risk factor

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Introduction

Preeclampsia is a condition unique to pregnancy, presenting with newly developed hypertension beyond 20 weeks of gestation and frequently associated with proteinuria, organ dysfunction in the mother, or restricted fetal growth.(1,2) Globally, it continues to be a major contributor to illness and death among mothers and newborns, accounting for up to 18% of maternal deaths and 40% of fetal deaths globally according to World Health Organization (WHO).(3,4) The incidence of preeclampsia is notably higher in developing countries, including Indonesia, where prevalence has reached 4.91%, with a case fatality rate of 24%.(5) Despite extensive research, the exact etiology of preeclampsia remains unclear. However, it is well-established that abnormal placentation, endothelial dysfunction, and an imbalance between angiogenic and anti-angiogenic factors, particularly elevated levels of soluble Fms-like tyrosine kinase-1 (sFlt-1), play a crucial role in its pathophysiology.(6,7) sFlt-1 functions as a circulating decoy receptor by attaching to vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), which decreases their availability and contributes to endothelial cell damage.(8)

Recent attention has focused on the epigenetic regulation of sFlt-1 expression, particularly DNA methylation of its promoter region. Hypomethylation of gene promoters is known to increase gene transcription, and studies suggest that hypomethylation of *sFlt-1* promoter may contribute to its overexpression in preeclampsia.(9,10) However, data regarding this mechanism in the Indonesian population remain limited. Therefore, this study was conducted to determine whether hypomethylation of sFlt-1 promoter and elevated expression of sFlt-1 in placental tissue are associated with the occurrence of preeclampsia.

Unlike previous studies that have primarily focused on Western or more generalized populations, this research specifically targets the high-risk, under-researched demographic in Bali. This study addresses the critical evidence gap by providing the first comprehensive molecular data on epigenetic and protein markers for preeclampsia in the Indonesian population, particularly in Bali. By focusing on this regional population, this study generates robust quantitative data that may enable the development of localized diagnostic strategies tailored to the specific needs of the region. This approach distinguishes our study from other researchers who have predominantly used broader, less context-specific methodologies, providing a unique perspective on preeclampsia risk factors in Southeast Asia.

Methods

Study Design and Subject Recruitment

A case-control study was conducted, and data on dependent and independent variables were collected at the same point in time. This study design focused on identifying associations rather than causality. This study was conducted at Prof. Dr. I.G.N.G. Ngoerah General Hospital, Denpasar, from April to December 2024, following approval by the Ethics Committee of the Faculty of Medicine, Universitas Udayana (Number: 0376/UN14.2.2.VII.14/LT/2024). Placental tissue samples were collected from postpartum women diagnosed with preeclampsia and from normotensive women who delivered at term. The inclusion criteria were singleton pregnancy, gestational age of ≥37 weeks, and absence of systemic disorders; while exclusion criteria included maternal infections, autoimmune diseases, and multiple pregnancies.

Subjects were recruited using consecutive sampling based on eligibility criteria. The sample size was determined using the formula for comparative proportions with a significance level of 0.05 and a power of 80%, resulting in a minimum of 30 subjects in each group. The independent variables for the multivariate analysis were selected based on previous literature and clinical relevance, including maternal age, body mass index (BMI), parity, and gene expression levels of *sFlt-1*. The logistic regression model was controlled for potential confounders including obesity, gestational age, and pre-existing conditions.

Tissue Collection and Preservation

Placental tissue (approximately 1 cm^3) was collected within 30 minutes after delivery from the maternal side of the placenta. Samples were immediately stored in RNAlater (Ambion, Austin, TX, USA) and transported to the Molecular Biology Laboratory, Universitas Udayana for further processing.

DNA Extraction and Methylation Analysis using Polymerase Chain Reaction (PCR)

Genomic DNA was isolated utilizing the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. The concentration and purity of the extracted DNA were then assessed using a NanoDrop spectrophotometer. Bisulfite modification of DNA was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). Methylation-specific PCR (MSP) was conducted to detect methylation

status of the *sFlt-1* promoter region using previously published primers and protocols.(11) The PCR products were analyzed by 2% agarose gel electrophoresis and visualized using UV transillumination. Quantification of *sFlt-1* promoter hypomethylation in placental tissue was performed using ImageJ (NIH, Bethesda, MD, USA) to analyze electrophoresis band intensity. The percentage of hypomethylation was calculated as: (Area of unmethylated band / Total area of methylated + unmethylated bands) × 100%.

RNA Extraction, Gene Expression Analysis, and Proteinuria Measurement

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and its concentration and integrity were assessed spectrophotometrically. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix (Applied Biosystems) with primers specific to *sFlt-1* and *GAPDH* as the housekeeping gene. The $2^{-\Delta\Delta Ct}$ method was used to quantify relative gene expression levels.(12)

In addition to gene expression analysis, proteinuria was measured to assess kidney function in the context of preeclampsia. Proteinuria, defined by the presence of ≥300 mg of protein in a 24-hour urine sample, is a critical diagnostic criterion for preeclampsia. It indicates endothelial dysfunction and renal damage, often linked to elevated sFlt-1 levels. Urine samples were collected from participants, and protein levels were quantified using the Bradford assay or dipstick method. The presence and severity of proteinuria were correlated with elevated sFlt-1 expression and promoter methylation status, contributing to the understanding of preeclampsia pathogenesis.

Statistical Analysis

Statistical analysis was performed using SPSS version 26.0 (IBM Corporation, Armonk, NY, USA). The normality of data was tested using the Kolmogorov–Smirnov test. Independent t-test or Mann–Whitney U test was used for group comparisons, depending on data distribution. Logistic regression was used for multivariate analysis to determine independent risk factors. A $p<0.05$ was considered statistically significant. Receiver operating characteristic (ROC) curve analysis was conducted to determine the diagnostic performance of methylation status and gene expression levels.

Results

Characteristics of Study Subjects

This study involved 60 pregnant women, who were divided into a case group of 30 women with preeclampsia and a control group of 30 normotensive women. The detailed comparison of characteristics including age, parity, and BMI between the groups is presented in Table 1. Based on normality tests, the data for age, parity, and BMI were found to be normally distributed ($p>0.05$).

Table 1. Relationship between subject characteristics and preeclampsia incidence.

Characteristics	Preeclampsia (n=30)	Non-preeclampsia (n=30)	OR	95% CI	*p*-value
Age (years), mean±SD	29.57±7.7	27.40±65.43			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 (%)					
Nulliparous	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			0.000[a,*]
BMI (kg/m^2), n (%)					
<18.5	0 (0)	1 (3,3)	9.33	2.84-30.60	0.000[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)			

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

The overall mean maternal age of the participants was 29.57±7.7 years old, while the overall mean parity was 1.73±1.28. There were no significant differences in the mean age (p=0.214) or mean parity (p=0.117) between the preeclampsia and non-preeclampsia groups. However, when age was analyzed as a categorical variable, maternal age of 35 years or older was found to significantly increase the risk of preeclampsia by 7-fold (OR: 7.00; 95% CI: 1.38–35.47; p=0.021). Categorizing parity into nulliparous and parous (parity≥1) revealed no significant association with the incidence of preeclampsia (p=0.117).

The overall mean BMI was 24.66±1.93 kg/m², and none of the subjects in the preeclampsia group were categorized as underweight (BMI<18.5 kg/m²). A comparison between groups showed that the mean BMI of women with preeclampsia was significantly higher than that of normotensive women (p<0.001). Subsequently, when BMI was categorized to assess the impact of obesity, it was found that obesity significantly increased the risk of developing preeclampsia (OR: 9.33; 95% CI: 2.84–30.60; p<0.001).

Hypomethylation of the *sFlt-1* Gene Promoter as a Risk Factor for Preeclampsia

Placental DNA was isolated and subjected to bisulfite conversion, followed by methylation-specific PCR (MSP) targeting the *sFlt-1* gene promoter region. The PCR products were assessed for methylation status using electrophoresis under UV light. Based on the band patterns observed, methylation status was classified into three categories: full methylation, indicated by the presence of only methylated bands; no methylation, shown by the presence of only unmethylated bands; and partial methylation, where both methylated and unmethylated bands were detected. These band patterns (Figure 1) were used to determine methylation status in relation to preeclampsia risk.

The median hypomethylation percentage in non-preeclamptic placentas was 77.50%, while in preeclamptic placentas it was 12.33% (Figure 2). As the data were not normally distributed, the Mann–Whitney U test was applied. The analysis indicated statistically significant differences in sFlt-1 promoter hypomethylation levels between the two groups (p<0.05), suggesting that hypomethylation of the sFLT-1 gene promoter was more prevalent in preeclamptic placental tissues.

The electrophoresis results were classified into three methylation patterns, with hypomethylation observed in 9 samples (15%), unmethylation detected in 12 samples (20%), and partial methylation identified in 39 samples (65%). To refine classification, a cut-off point was determined using ROC curve analysis. The optimal cut-off value was 20.5%, yielding an area under curve (AUC) of 0.763, sensitivity of 76.3%, and specificity of 62.1% (Figure 3). This cut-off allowed further classification of partial methylation into functional categories (hypomethylated *vs.* unmethylated). Hypomethylation of the *sFlt-1* gene promoter was more prevalent in the preeclampsia group (76.6%) than in the non-preeclampsia group (26.7%). Chi-square analysis showed a significant association, with hypomethylation increasing the risk of preeclampsia by 9-fold (OR: 9.0; 95% CI: 2.8–29.1; p=0.000) (Table 2).

There was a robust association between hypomethylation status and preeclampsia risk, with the hypomethylated individuals having a 9-fold increased likelihood of developing the condition. This significant finding underscores the potential of *sFlt-1* promoter hypomethylation as a powerful predictor for preeclampsia, suggesting that it may serve as a valuable biomarker for early detection and risk stratification in high-risk pregnancies.

High sFlt-1 Expression as a Risk Factor for Preeclampsia

The normality test showed that sFlt-1 expression data were not normally distributed (p<0.001), so the Mann–Whitney test was used. Results revealed a significantly higher sFlt-1 expression in the preeclampsia group compared to the

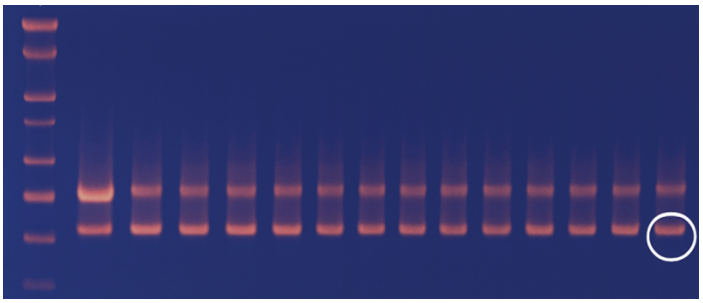

Figure 1. PCR amplification for confirmation of methylation status. The circled band in the control lane (C) indicates an absence of the methylated DNA. S: sample; C: control.

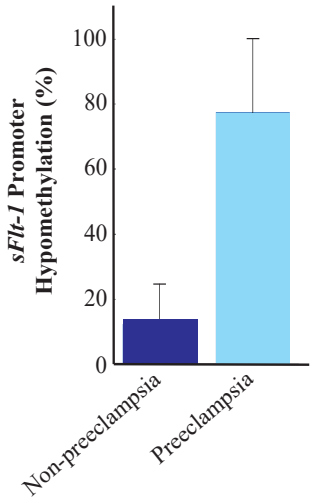

Figure 2. Comparison of *sFlt-1* promoter hypomethylation (%) between placental tissue from women with preeclampsia (n=30) and non-preeclampsia (n=30). Values are shown in median and interquartile range. A Mann–Whitney U test revealed a statistically significant difference between the two groups ($p<0.05$), indicating significantly lower hypomethylation levels in the preeclampsia group.

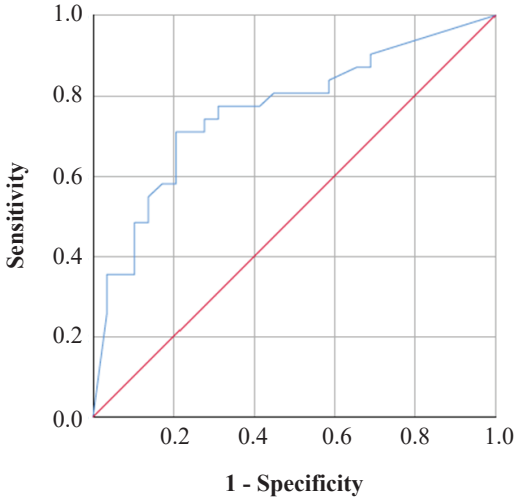

Figure 3. ROC curve analysis of the percentage of *sFlt-1* promoter DNA hypomethylation for predicting preeclampsia incidence. The ROC curve revealed an optimal cut-off value of 20.5% for the percentage of *sFlt-1* promoter DNA hypomethylation, demonstrating an AUC of 0.763. This cut-off value exhibited a sensitivity of 76.3% and a specificity of 62.1%, indicating its moderate diagnostic performance for predicting preeclampsia.

control group (4.5±5.2 *vs.* 2.6±3.2; $p<0.001$), as illustrated in Figure 4. This indicated that elevated sFlt-1 expression is significantly associated with preeclampsia.

Furthermore ROC curve analysis was performed to determine the optimal cut-off of sFlt-1 expression to predict the occurrence of preeclampsia (Figure 5). The cut-off value of sFlt-1 expression with the best sensitivity and specificity values was obtained at 3.6 pg/mL. This cut-off value has an AUC value of 0.788 with a sensitivity of 76% and a specificity of 70%. Values of 3.6 pg/mL or lower were categorized as low expression, while values above 3.6 pg/mL were categorized as high expression. This threshold provides valuable insight into the potential for using sFlt-1 expression levels in clinical practice for early detection and risk stratification of preeclampsia. As shown in Table 3, 73.4% of preeclamptic women exhibited high sFlt-1 expression levels (>3.6 pg/mL), in contrast to only 30.0% in the non-preeclamptic group. Statistical analysis confirmed that high sFlt-1 expression significantly increased the risk of preeclampsia (OR: 2.57; 95% CI 1.36–4.83; $p=0.001$). These findings suggested that elevated sFlt-1 expression may serve as a clinically relevant marker for identifying women at increased risk of preeclampsia.

Multivariate Analysis of Preeclampsia Risk Factors

To further investigate the independent contribution of various factors to the risk of preeclampsia, a multivariate analysis was conducted using logistic regression. This analysis included *sFlt-1* gene promoter hypomethylation and high *sFlt-1* expression as primary variables of interest, while controlling for potential confounding variables such as maternal age, parity, and BMI. The results, summarized in Table 4, demonstrated that obesity, defined as a BMI greater than 25 kg/m², significantly increased the risk of preeclampsia by 11.15 times (AOR: 11.15; 95% CI: 2.01–61.78; $p=0.006$). In addition, high *sFlt-1* expression (>3.6 pg/mL) was found to independently elevate the risk by 12.55 times (AOR: 12.55; 95% CI: 1.95–80.83; $p=0.008$). Notably, hypomethylation of the *sFlt-1* promoter (<20.5%) emerged as the strongest predictor, with a 21.18-

Table 2. Relationship between *sFlt-1* promoter DNA hypomethylation and the incidence of preeclampsia.

sFlt-1 Gene Promoter Methylation	Preeclampsia (n=30)	Non- preeclampsia (n=30)	OR	95% CI	*p*-value
Hypomethylation (<20.5%)	23 (76.6%)	8 (26.7%)	9.0	2.8-29.1	0.000*
Unmethylation (≥20.5%)	7 (23.4%)	22 (73.3%)			

Tested with Mann–Whitney test; *Significant with $p<0.05$.

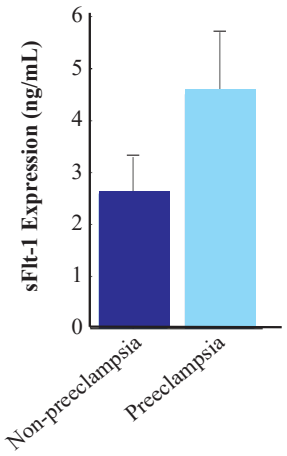

Figure 4. Comparison sFlt-1 levels in placental tissue between women with preeclampsia (n=30) and non-preeclampsia (n=30). Values are shown in median and interquartile range. A Mann–Whitney U test revealed a statistically significant difference between the two groups (p<0.05), indicating significantly lower expression of sFlt-1 in the preeclampsia group.

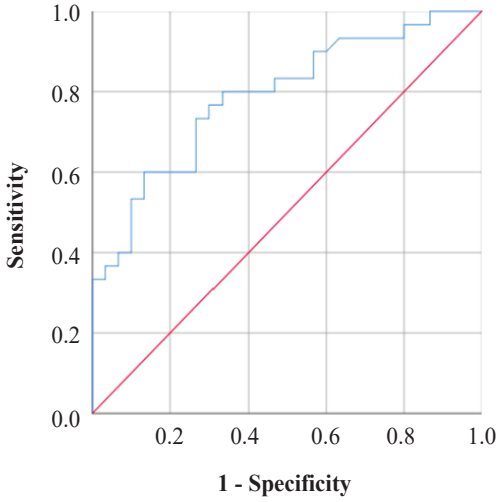

Figure 5. ROC curve analysis of sFlt-1 expression for predicting preeclampsia incidence. The ROC curve revealed an optimal cut-off value of 3.6 pg/mL for sFlt-1 expression, demonstrating an AUC of 0.788. This cut-off value exhibited a sensitivity of 76% and a specificity of 70%, indicating its moderate diagnostic performance for predicting preeclampsia.

fold increased risk of developing preeclampsia (AOR: 21.18; 95% CI: 2.49–179.72; p=0.005). However, the wide confidence intervals highlight variability in the results, suggesting that these findings should be interpreted with caution. Given the small sample size and potential for bias, further validation in larger cohorts is needed to confirm the robustness of this association.

Conversely, maternal age >35 years and nulliparity were not significantly associated with preeclampsia in this model. Maternal age >35 years yielded an AOR of 3.36 (95% CI: 0.40–23.91; p=0.310), while nulliparity had an AOR of 0.55 (95% CI: 0.070–4.370; p=0.576). The lack of statistical significance and wide confidence intervals that included the null value (1.0) suggest that these factors did not contribute independently to preeclampsia risk in the studied population. Hence, this multivariate analysis highlights that obesity, elevated sFlt-1 expression, and hypomethylation of the *sFlt-1* promoter were significant and independent risk factors for preeclampsia. These findings underscore the potential value of molecular biomarkers in predicting preeclampsia and guiding early intervention strategies.

Discussion

DNA methylation, an epigenetic mechanism involving the addition of methyl groups to CpG dinucleotides, typically silences gene expression when occurring in promoter regions.(13) In this study, significant hypomethylation of the *sFlt-1* promoter in the preeclampsia group was found, which was associated with increased *sFlt-1* mRNA expression. The *sFlt-1* gene encodes a soluble receptor that binds VEGF and PlGF, inhibiting angiogenesis. Its overexpression disrupts placental and maternal vascular balance, contributing to endothelial dysfunction and clinical symptoms of preeclampsia, including hypertension and fetal growth restriction.(14) The current findings align with the established pathophysiology of preeclampsia.(15)

The reuslts of this study is also align with earlier studies reporting hypomethylation at specific CpG sites in the *sFlt-1* promoter in placental tissue from preeclamptic pregnancies, which negatively correlated with systolic blood pressure and proteinuria severity.(11) This methylation pattern shows

Table 3. Relationship of sFLT-1 expression to preeclampsia incidence.

sFlt-1 Expression	Preeclampsia (n= 30)	Non- preeclampsia (n= 30)	OR	95% CI	*p* -value
Low (≤3.6 pg/mL)	8 (26.6%)	21 (70.0%)	2.57	1.36-4.83	0.001*
High (>3.6 pg/mL)	22 (73.4%)	9 (30.0%)			

Tested with Mann–Whitney test; *Significant with p<0.05.

Table 4. Multivariate analysis for risk factors preeclampsia.

Risk Factors	*Adjusted* OR	95% CI	*p*- value
Age >35 years	3.36	0.40-23.91	0.310
Nulliparous	0.55	0.070-4.370	0.576
Obesity (BMI >25 kg/m^2)	11.15	2.01-61.78	0.006*
High sFlt-1 expression (>3.6 pg/mL)	12.55	1.95-80.83	0.008*
sFlt-1 DNA hypomethylation (<20.5 %)	21.18	2.49-179.72	0.005*

Tested with logistic regression;*Significant with $p<0.05$.

promise as a screening tool, with an AUC of 0.788, 76% sensitivity, and 70% specificity, highlighting its potential for early diagnosis and risk stratification. Implementing this cut-off in clinical settings may enable earlier identification and personalized management of high-risk pregnancies. Similar patterns of reduced methylation in angiogenesis-related genes, including *Flt-1*, that have been observed in early-onset preeclampsia.(16) Supporting this mechanism, a hypoxia-induced preeclampsia mouse model demonstrated placental hypoxia-induced hypomethylation of genes such as *Flt-1* and also increased sFlt-1 expression (17), consistent with the known role of hypoxia in upregulating sFLT-1 via hypoxia-inducible factor (HIF)-1α signaling (18).

Furthermore, recent Epigenome-wide Association Studies (EWAS) have shown that accelerated epigenetic aging in placental tissues is linked to preeclampsia, supporting the idea that the DNA methylation patterns reflect not only genetic regulation but also environmental insults, such as oxidative stress and hypoxia.(19) This study highlights the potential of *sFlt-1* methylation as a biomarker for preeclampsia. However, due to the small sample size and retrospective design, its clinical application remains exploratory. First, this study shows that hypomethylation of the *sFlt-1* gene contributes to its overexpression, clarifying its role in preeclampsia. Second, it supports the use of *sFlt-1* methylation status as a potential biomarker for early prediction or risk stratification. Third, since DNA methylation is reversible, therapies targeting epigenetic regulation may help control abnormal gene expression in high-risk pregnancies.(20)

From a translational perspective, a future direction would be to investigate whether circulating cell-free DNA methylation patterns in maternal blood reflect the placental methylation status of *sFlt-1*. This could enable non-invasive testing for early detection of preeclampsia. Additionally, it would be valuable to assess how environmental exposures (*e.g.*, diet, pollutants, hypoxia) might influence *sFlt-1* methylation during pregnancy, and whether these effects differ based on genetic background. However, the current study has some limitations. Although methylation and mRNA expression were measured, sFlt-1 protein levels in maternal serum or placental tissue were not assessed. Moreover, the cross-sectional design does not allow us to determine whether hypomethylation precedes the development of preeclampsia or is a consequence of the disease process. As sFlt-1 expression changes throughout gestation, the cut-off values found here may not reflect optimal thresholds for predicting risk in the second or third trimester. This study also only included 60 pregnant women from a single center, introducing potential sampling bias.

While obesity was found to be an independent risk factor for preeclampsia, it remains unclear whether it exerts its effect partially through modulating *sFlt-1* methylation or expression levels. Future studies exploring these potential mechanistic links may enhance our understanding of how maternal clinical characteristics, such as obesity, influence molecular markers like sFlt-1 in the pathogenesis of preeclampsia. This could provide valuable insights into the molecular pathways that underlie clinical risk factors, bridging the clinical and molecular dimensions of the disease. This approach provided a more robust understanding of the relationship between sFlt-1 and preeclampsia, making the findings more reliable. This study also provides novel insight into the epigenetic dysregulation of sFLT-1 in preeclampsia by demonstrating, for the first time in Bali, Indonesia, a significant association between hypomethylation of the *sFLT-1* promoter region in placental tissue and elevated placental sFLT-1 expression, esthablishing it as a key molecular risk factor for the disorder.

Conclusion

This study concludes that hypomethylation of the *sFlt-1* gene promoter region is significantly associated with an increased risk of preeclampsia. Additionally, elevated expression of sFlt-1 in placental tissue was found to be an independent risk factor for the development of the disease.

These findings indicate that epigenetic regulation of angiogenic factors plays a crucial role in the pathogenesis of preeclampsia. The *sFlt*-1 methylation status and expression level of sFlt-1 may serve as promising biomarkers for early detection and potential targets for therapeutic intervention in preeclamptic pregnancies.

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Authors Contribution

VSPD performed the data collection and conduct the research. IMD supervised and gave guidance throughout the study. IGMP, AS, and ESP provided insightful feedback and expert knowledge that significantly enriched this manuscript. AANJY performed the data analysis and prepared the original manuscript draft; while AANJY and W revised and edited 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.

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