


DNA Methylation of Endoglin Pathway Genes in Pregnant Women With and Without Preeclampsia

Allison H Rietze¹, Yvette P Conley^{1,2}, Dianxu Ren³,
Cindy M Anderson⁴, James M Roberts^{5,6,7,8}, Arun Jeyabalan^{5,6,8},
Carl A Hubel^{5,6} and Mandy J Schmella¹

¹Department of Health Promotion and Development (School of Nursing), University of Pittsburgh, Pittsburgh, PA, USA. ²Department of Human Genetics (Graduate School of Public Health), University of Pittsburgh, Pittsburgh, PA, USA. ³Department of Health and Community Systems (School of Nursing), University of Pittsburgh, Pittsburgh, PA, USA. ⁴Martha S Pitzer Center for Women, Children, and Youth (College of Nursing), The Ohio State University, Columbus, OH, USA. ⁵Department of Obstetrics, Gynecology, & Reproductive Sciences (School of Medicine), University of Pittsburgh, PA, USA. ⁶Magee-Womens Research Institute, Pittsburgh, PA, USA. ⁷Department of Epidemiology (Graduate School of Public Health), University of Pittsburgh, Pittsburgh, PA, USA. ⁸Clinical and Translational Science Institute, University of Pittsburgh, Pittsburgh, PA, USA.

Epigenetics Insights
Volume 13: 1–7
© The Author(s) 2020
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/2516865720959682


ABSTRACT

OBJECTIVE: We compared blood-based DNA methylation levels of endoglin (*ENG*) and transforming growth factor beta receptor 2 (*TGFβR2*) gene promoter regions between women with clinically-overt preeclampsia and women with uncomplicated, normotensive pregnancies.

METHODS: We used EpiTect Methyl II PCR Assays to evaluate DNA methylation of CpG islands located in promoter regions of *ENG* (CpG Island 114642) and *TGFβR2* (CpG Island 110111). Preeclampsia was diagnosed based on blood pressure, protein, and uric acid criteria. N=21 nulliparous preeclampsia case participants were 1:1 frequency matched to N=21 nulliparous normotensive control participants on gestational age at sample collection (± 2 weeks), smoking status, and labor status at sample collection. Methylation values were compared between case and control participant groups [(*ENG* subset: n=20 (9 cases, 11 controls); *TGFβR2* subset: n=28 (15 cases, 13 controls)].

RESULTS: The majority of the preeclampsia cases delivered at ≥ 34 weeks' gestation (83%). Average methylation levels for *ENG* ([M \pm (SD)]; Case Participant Group = $6.54\% \pm 4.57$ versus Control Participant group = $4.81\% \pm 5.08$; $P = .102$) and *TGFβR2* (Case Participant Group = $1.50\% \pm 1.37$ vs Control Participant Group = $1.70\% \pm 1.40$; $P = .695$) promoter CpG islands did not differ significantly between the participant groups. Removal of 2 extreme outliers in the *ENG* analytic subset revealed a trend between levels of *ENG* methylation and pregnancy outcome (Case Participant Group = $5.17\% \pm 2.16$ vs Control Participant Group = $3.36\% \pm 1.73$; $P = .062$).

CONCLUSION: Additional epigenetic studies that include larger sample sizes, investigate preeclampsia subtypes, and capture methylation status of CpG island shores and shelves are needed to further inform us of the potential role that *ENG* and *TGFβR2* DNA methylation plays in preeclampsia pathophysiology.

KEYWORDS: Methylation, pregnancy, pre-eclampsia

RECEIVED: May 9, 2020. **ACCEPTED:** August 25, 2020.

TYPE: Original Research

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Institutes of Health [T32NR009759, F32NR014622, P01HD30367]; and the Preeclampsia Foundation.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

CORRESPONDING AUTHOR: Mandy J Schmella, Department of Health Promotion and Development (School of Nursing), University of Pittsburgh School of Nursing, 3500 Victoria Street, 440 Victoria Building, Pittsburgh, PA 15261, USA. Email: mjb111@pitt.edu

Introduction

Preeclampsia is a multi-system, hypertensive pregnancy-specific syndrome that affects approximately 3% to 5% of pregnancies,^{1,2} and remains a significant cause of morbidity, mortality, and poor later-life health outcomes for both the mother and the infant.³ Although preeclampsia is a significant cause of maternal and infant morbidity and mortality, the pathophysiology is not completely understood, and no clinically useful biomarkers for the prediction and prevention of preeclampsia have been identified.⁴⁻⁶ At present, management is based on serial assessment of maternal and

fetal wellbeing, treatment of associated signs/symptoms (eg, antihypertensive therapy for management of acute onset severe range blood pressure), and delivery at ≥ 34 weeks' or ≥ 37 weeks' gestation for women with and without evidence of severe features of preeclampsia, respectively, unless earlier delivery is indicated.³

Placental dysfunction plays a key role in the development of preeclampsia, as it creates an environment characterized by intermittent hypoxia. In the setting of intermittent hypoxia, it is believed that the placenta becomes distressed (eg, oxidative and syncytiotrophoblast stress), thereby setting the stage for placental



damage, release of placenta debris into the maternal circulation, and an unfavorable maternal response to these factors.⁷

A growing body of evidence supports a biologically plausible role for endoglin (ENG), including its placentally-derived soluble counterpart (sENG), and other endoglin-related genes. Endoglin is a multi-functional, trans-membrane co-receptor of the transforming growth factor beta family (TGF β) that is expressed in a variety of cell types, including placental syncytiotrophoblasts and vascular endothelial cells.⁸⁻¹⁰ As mentioned above, it is believed that hypoxia creates a stressful placental milieu, thereby promoting placental damage and release of placental debris into the maternal circulation. Hypoxia is one of many factors that stimulates endoglin expression,¹¹ and in preeclampsia, the expression of ENG mRNA is increased in the cellular component of maternal blood across all 3 trimesters of pregnancy, as well as in first and third trimester placental samples.¹²⁻¹⁵ Moreover, the soluble form of endoglin (sENG), which is part of the placental debris, is also elevated in the maternal circulation of women who develop preeclampsia.¹⁶⁻¹⁸ Once in the maternal circulation, sENG is thought to interfere with downstream signaling along the TGF β pathway, including the regulation of vascular tone.¹⁸ Third, using a tagging single nucleotide polymorphism approach (tSNP), it has been previously shown that maternal genetic variation in the endoglin pathway, including the *ENG* and *TGF β 2* receptors, has been implicated in susceptibility to/protection from preeclampsia; however, mechanisms that may explain these associations have not been defined.^{19,20}

DNA methylation is a form of epigenetic regulation that can greatly affect gene expression and is a potential molecular mechanism that may explain modification of the endoglin pathway, including the genetic association between the ENG pathway, and susceptibility to/protection from preeclampsia. In a small pilot study, Anderson et al, detected differences in blood-based, genome-wide DNA methylation profiles during the first trimester in women who later developed preeclampsia compared to women who had uncomplicated pregnancies; however, methylation differences were not detected specifically in the 2 endoglin pathway candidate genes (*ENG* and *TGF β 2*) that were investigated in our study.²¹ Several other studies have also evaluated DNA methylation profiles in maternal peripheral white blood cells using both candidate gene and genome-wide approaches.²²⁻²⁵ These studies have shown that different genes display differences in DNA methylation in women with preeclampsia as compared to normotensive women, including genes that are hypomethylated and hypermethylated in preeclampsia. However, with respect to DNA methylation in maternal peripheral blood as it relates to preeclampsia, there is a lack of research specifically looking at methylation levels of endoglin pathway genes. The purpose of this study was to compare blood-based DNA methylation levels of *ENG* and *TGF β 2* gene promoter regions between women with clinically-overt preeclampsia and women with uncomplicated, normotensive pregnancies. Gene promoter regions were selected as the target for our interrogation, as methylation status

of these CpG rich islands are known to influence gene transcription.

Methods

Participants and phenotype designations

This study was reviewed and approved by the University of Pittsburgh Human Research Protection Office. Case and control participants were previously enrolled in the Prenatal Exposures and Preeclampsia Prevention (PEPP) cohort study (parent study) conducted at Magee-Womens Hospital of the University of Pittsburgh Medical Center, and provided consent for the sharing of their de-identified samples/data. Pregnant women between the ages of 14 and 44 years were recruited/enrolled into the PEPP study at 20 weeks' gestation, or earlier, and were followed through delivery, or they were recruited cross-sectionally after admission due to suspected preeclampsia (parent study data and samples were collected between 1997 and 2007). Pregnant women were excluded from participation in the PEPP study if they had a history of chronic renal disease, hypertension, diabetes, multi-fetal gestation, infection, or metabolic disorders—all of which are associated with an increased risk for preeclampsia. The sample for this study was restricted to White participants based on the relationship between genetic variation in *ENG* and preeclampsia in White women documented in previous work.¹⁹ Although an association between *TGF β 2* genetic variation and preeclampsia across different ethnicities has been demonstrated,^{19,20} it was not feasible to include these various ethnicities in this study. All samples and demographic/clinical data were provided to the investigators in a de-identified manner. Due to the fair amount of evidence indicating that long term storage of DNA does not impact the stability of DNA methylation,^{26,27} we utilized these bio-banked samples to collect and analyze DNA methylation between May 2017 and April 2018.

Case participants had clinically-overt preeclampsia when the peripheral blood samples were collected. The diagnosis of preeclampsia was based on a research definition: (1) blood pressure ≥ 140 mmHG systolic and/or 90 mmHg diastolic after 20 weeks' gestation based on the average of the 4 most recent blood pressures taken in the hospital prior to therapeutic intervention; (2) proteinuria ≥ 300 mg/24 hours, ≥ 0.3 protein/creatinine ratio, $\geq 2+$ on a random urine specimen, or $\geq 1+$ on a catheterized urine specimen; and (3) hyperuricemia with serum uric acid concentration ≥ 1 standard deviation from normal for gestational age.²⁸ All cases diagnosed with preeclampsia had live births. Control participants were defined as women who remained normotensive and non-proteinuric throughout pregnancy, and delivered healthy term babies.

DNA extraction, DNA quantification, and DNA methylation data collection

Genomic DNA was extracted via protein precipitation from maternal peripheral blood samples that were collected during the third trimester prior to delivery (case participants: all within

a week of delivery [$\bar{X} = 0.10 \pm 0.11$] control participants: 0 to 8 weeks prior to delivery [$\bar{X} = 2.15 \pm 3.04$]). Genomic DNA quantity and quality were evaluated at the University of Pittsburgh Genomics Research Core. Methylation data were collected using the following EpiTect Methyl II PCR Assays: Human *ENG* (CpG Island ID 114642; CpG Island Location: Chr9[130616540-130617120]; Assay catalogue number: EPHS114642-1A) and Human *TGF β 2* (CpG Island ID 110111; CpG Island Location: Chr3[30647816-3064881]; Assay catalogue number: EPHS110111-1A) (Qiagen® Inc., Germantown, Maryland). These commercially available assays allow for interrogation of methylation status across a distinct CpG-rich sequence, also known as a CpG island, located in the promoter region of candidate genes.²⁹ CpG islands represent clusters of “C” and “G” nucleotides, and when they are methylated in the promoter region of a gene, they are associated with repression of gene expression.³⁰ Each distinct EpiTect Methyl II PCR assay uses a restriction enzyme approach, followed by real-time PCR, to quantify the methylation status for the selected CpG island in the promoter region of interest.²⁹ The assay primers are commercially designed to amplify a single CpG island within the candidate gene promoter region, which is defined as 5 kb upstream to 3 kb downstream of the gene’s transcription start site. Both candidate gene assays were designed by Qiagen® from the NCBI Homo Sapiens Build Number 36, version 2, and PCR primer sequences are not provided by the vendor.²⁹ All participant samples were run in duplicate for both the *ENG* and *TGF β 2* assays. Only samples that had 2 concordant values ($|\text{Run 1 value (\% methylated)} - \text{Run 2 value (\% methylated)}| \leq 15\%$ different for both *ENG* and *TGF β 2*), were included in the final analysis, with the average of these 2 values used for analysis.

Final sample size and statistical analysis

For this study, we originally performed 1:1 frequency matching of $n=21$ self-reported White women with clinically-overt preeclampsia to $n=21$ self-reported White women with uncomplicated, normotensive pregnancies on the following characteristics: gestational age that the peripheral blood sample was collected (± 2 weeks), nulliparity, smoking status, and labor status during sample collection. We recognize that there are numerous other covariates to consider, including maternal BMI and family history; however, we were unable to control for all potential covariates due to small sample size.

After re-review of the phenotype data used by the parent study to determine pregnancy outcome, one case participant was found to be misclassified (didn’t meet either case or control phenotype criteria), and was later omitted from the analyses. This omission resulted in a final sample size of $n=41$ ($n=20$ case participants and $n=21$ control participants) that were available for methylation data collection. Methylation data were collected in duplicate and reviewed for the 41 samples. Upon reviewing the methylation data, we identified samples

that either (1) failed both of the methylation data collection rounds [*ENG* $n=0$; *TGF β 2* $n=8$], (2) generated data for only one round of methylation data collection [*ENG* $n=9$; *TGF β 2* $n=2$], (3) or generated discordant data values that differed by more than 15% (eg, Sample X: methylation value for run 1 (1.11%) vs methylation value for run 2 (55.2%) [*ENG* $n=12$; *TGF β 2* $n=3$]). Because of our stringent and conservative approach to assessing data quality, those samples identified as meeting 1 of the 3 criteria above were omitted from the analysis. This resulted in a final analytic subset that included 20 participants (9 case participants, 11 control participants) for *ENG* methylation analysis, and 28 participants (15 case participants, 13 control participants) for *TGF β 2* methylation analysis.

Data were analyzed using IBM’s SPSS Statistics Version 24 software (IBM Corp., Armonk, New York). Independent samples *t*-tests and Mann-Whitney *U* tests were used to compare demographic and clinical characteristics between case and control group participants. The Mann-Whitney *U* test was used to compare methylation levels of *ENG* and *TFG β 2* between case and control group participants. Due to the small sample size, analyses were not adjusted for covariates, such as smoking status. Moreover, analyses were not adjusted for cell type heterogeneity, as we did not collect epigenome-wide data that are required to estimate cell proportions.

Results

Demographic/clinical characteristics

A side-by-side comparison of demographic and clinical characteristics for the case and control participant groups included in the *ENG* and *TFG β 2* methylation analyses, respectively, is provided in Table 1. Although we were unable to include all participants in our final analyses, which could have influenced our 1:1 frequency matching, the case and control participant groups that we analyzed remained similar with respect to our matching criteria. All participants included in the analyses were nulliparous and self-reported their race as White. The majority of participants were non-smokers. For both analytic sets, gestational age at sample collection and delivery was similar between case and control participant groups. All control participants delivered at term, and the majority of case participants delivered at term (cases delivering at ≥ 34 weeks: *ENG* group (78%) & *TFG β 2* (87%). Consistent with preeclampsia phenotype by definition, average blood pressure measurements in labor were significantly higher in case participants compared to control participants. Plasma sENG levels measured in the third trimester were significantly higher in the case participant group compared to control participant group (Figure 1), which was in alignment with results previously reported in a landmark article by Levine et al.¹⁶ Plasma sENG levels were not significantly correlated with *ENG* methylation levels (Spearman’s $\rho=0.27$, $P=.25$). In the *TFG β 2* analytic set, pre-pregnancy BMI (calculated based on self-reported pre-pregnancy weight and measured height at first prenatal visit) was significantly higher in the case participant

Table 1. Demographic and clinical characteristics.

CHARACTERISTICS	ENG METHYLATION ANALYSIS			TGFB β R2 METHYLATION ANALYSIS		
	CASES (N=9)	CONTROLS (N=11)	P	CASES (N=15)	CONTROLS (N=13)	P
Maternal age, years (M (SD))	29.15 (5.14)	27.30 (6.82)	.510 ^a	28.12 (6.64)	27.51 (5.24)	.790 ^a
Gestational age at delivery, weeks (M (SD))	37.64 (3.58)	39.05 (1.07)	.704 ^b	37.79 (2.78)	39.29 (1.24)	.221 ^b
Gestational age at sample collection, weeks (M (SD))	37.51 (3.62)	36.40 (3.30)	.254 ^b	37.68 (2.85)	36.81 (3.53)	.221 ^b
Average SBP <20 wks, mmHg (M (SD))	119.13 (9.45)	113.82 (8.85)	.227 ^a	115.20 (9.58)	113.54 (8.84)	.639 ^a
Average DBP <20 wks, mmHg (M (SD))	74.88 (4.97)	68.82 (5.08)	.019 ^a	71.93 (5.05)	67.92 (6.41)	.076 ^a
Average SBP in labor, mmHg (M (SD))	152.22 (9.24)	122.18 (11.42)	$P<.001^a$	151.53 (10.62)	116.92 (10.87)	$P<.001^a$
Average DPB in labor, mmHg (M (SD))	93.33 (8.03)	71.27 (8.90)	$P<.001^a$	89.60 (5.60)	70.23 (8.80)	$P<.001^a$
Pre-pregnancy BMI, kg/m ² (M (SD))	30.04 (8.07)	25.58 (7.65)	.149 ^b	29.52 (7.37)	23.91 (4.61)	.020 ^b
Average plasma sENG, ng/mL (M (SD))	30.84 (20.60)	10.55 (5.58)	.004 ^b	31.28 (22.32)	9.18 (3.49)	$P<.001^b$
Smoking status (n (%)): No	5 (55.56%)	7 (63.64%)	.714 ^c	8 (53.33%)	8 (61.54%)	.662 ^c
Sample collected in labor (n (%)): No	7 (77.78%)	8 (72.73%)	.795 ^c	9 (60.0%)	8 (61.54%)	.934 ^c

Abbreviations: M (SD), mean (standard deviation); SBP, systolic blood pressure; DBP, diastolic blood pressure; mmHg, millimeters of mercury; BMI, body mass index.

^aIndependent samples *t*-test.

^bMann-Whitney *U* test.

^cPearson Chi-Square test.

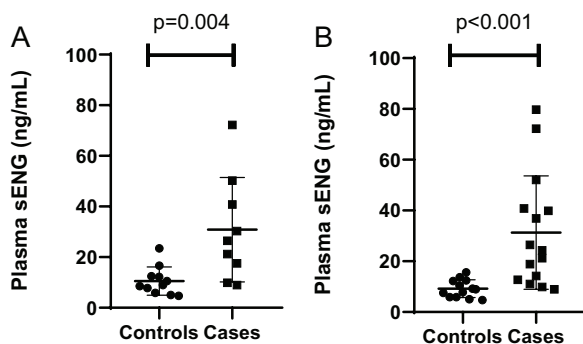


Figure 1. Plasma sENG concentrations stratified by case/control status. (A) Plasma sENG concentrations from participants included in the *ENG* methylation analysis. (B) Plasma sENG concentrations from participants included in the *TGFB β R2* methylation analysis. Black bars represent the mean and standard deviation.

group compared to control participant group, but this difference was not seen in the *ENG* analytic set.

ENG and TGFB β R2 Methylation Results

Evaluation of the CpG island located in the promoter region of *ENG* revealed that DNA methylation levels were higher in the case participant group compared to control participant group,

but this difference was not statistically significant (Table 2, Figure 2). Furthermore, the CpG island located in the promoter region of *TGFB β R2* was not differentially methylated between the participant groups (Table 2, Figure 2). For *ENG*, the minimum and maximum percent methylated value among the case participants was 2.24% and 17.46%, respectively. Among control participants, the minimum and maximum percent methylated value for *ENG* was 1.65% and 19.30%, respectively.

Upon further visual inspection of *ENG* methylation data distribution, there appeared to be 2 extreme outliers (case value of 17.46% and control value of 19.30%). Both of these participants were smokers, and their sENG levels were 9.90 ng/uL (case participant) and 4.71 ng/uL (control participant). To explore the potential influence of these 2 values, they were omitted from analysis. DNA methylation levels remained higher in case participant group compared to the control participant group, but the difference was not statistically significant ($P=.062$). For *TGFB β R2*, the minimum and maximum percent methylated value among case participants was 0.01% and 5.24%, respectively. Among control participants, the minimum and maximum percent methylated value for *TGFB β R2* was 0.19% and 4.22%, respectively. Upon further review of the *TGFB β R2* methylation data distribution, 2 potential outliers were identified (control participant value of 4.22% and case

Table 2. ENG and TGFβR2 DNA methylation.

		Cases (n=9)	Controls (n=11)	P value
ENG	M (SD)	6.54 (4.57)	4.81 (5.08)	
% Methylated	Median (IQR)	5.20 (3.57, 7.86)	2.72 (2.38, 6.04)	.102 ^a
		Cases (n=15)	Controls (n=13)	P value
TGFβR2,	M (SD)	1.50 (1.37)	1.70 (1.40)	
% Methylated	Median (IQR)	1.50 (0.20, 2.12)	1.51 (0.48, 2.64)	.695 ^a

Abbreviations: M (SD), mean (standard deviation); Median (IQR), median (inter-quartile range).

^aMann-Whitney *U* test.

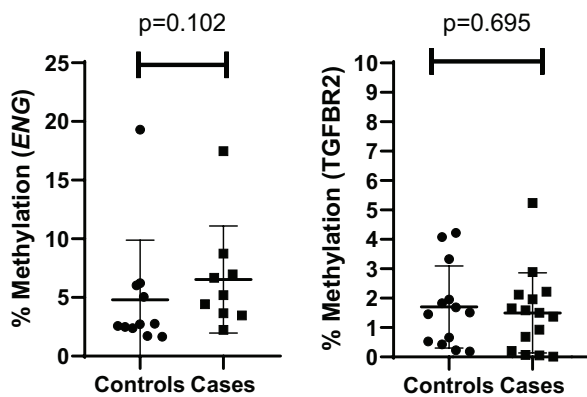


Figure 2. Endoglin and transforming growth factor beta receptor 2 methylation distributions stratified by case/control status. Black bars represent the mean and standard deviation.

participant value of 5.24%), but removal of these values from the analysis did not alter the results ($P = .681$).

Discussion/Conclusion

This study was designed to investigate if blood-based DNA methylation levels of *ENG* and *TGFβR2* gene promoter regions differ significantly between women with clinically-overt preeclampsia compared to normotensive pregnant women, as methylation may represent a potential mechanism that explains the endoglin pathway's role in preeclampsia. Neither of the CpG islands within the promoter regions of *ENG* and *TGFβR2* demonstrated statistically significant differences in methylation status between women with clinically-overt preeclampsia and women with normotensive, uncomplicated pregnancies; however, methylation levels of the *ENG* CpG island 114642 were higher in the case participant group compared to the control participant group. Omission of 2 potential extreme outliers did not reveal a statistically significant difference.

Gene expression is regulated by many mechanisms, including DNA methylation. DNA methylation of gene promoter regions is typically associated with repression of gene expression.³⁰ In previous studies, *ENG* mRNA expression has been found to be elevated in the cellular component of maternal blood, in women with or destined to develop preeclampsia, throughout pregnancy.^{12,14,15} Based on these findings, one

would expect that the level of *ENG* promoter methylation would be lower in women with or destined to develop preeclampsia compared to normotensive controls. Yet, in our study, we found that *ENG* promoter methylation levels were actually higher in women with preeclampsia. Unfortunately, we were unable to collect gene expression data for these samples to further investigate the relationship between *ENG* promoter methylation levels and *ENG* mRNA expression in our sample. Overall, these findings could indicate that there is no relationship between methylation of the *ENG* and *TGFβR2* promoters and clinically-overt preeclampsia, or these findings could be influenced by a lack of power that would be needed to detect statistically significant differences when they truly existed. Moreover, the relationship may only be relevant to certain preeclampsia subtypes, such as early-onset preeclampsia, which was not adequately represented in our sample.

When designing epigenetic studies, there are important factors to consider that can affect the collection and interpretation of methylation data: (1) tissue type, (2) cell type heterogeneity, (3) gene coverage, and (4) environment/demographic/clinical characteristics. Methylation levels differ by tissue type and most studies related to methylation profiles in preeclampsia have been conducted in the placenta, with few exploring methylation in the blood. Blood is not the most proximal tissue to study for this phenotype; however, identifying a biomarker for preeclampsia in the blood would advance the detection and management of this disease because blood is an accessible tissue. While previous studies conducted with white blood cells have demonstrated a difference in DNA methylation between women with preeclampsia and normotensive women, a limitation of these previous studies, as well as our study, includes not correcting for cell type heterogeneity.^{21,23,24} It is known that white blood cell proportions change across pregnancy and differ among women with and without preeclampsia, due to the inflammatory response present during normal pregnancy that is exaggerated during preeclampsia.³¹⁻³³ As such, not controlling for this could influence the interpretation/validity of results.

This study was limited to the evaluation of the single CpG island EpiTect Methyl II PCR assays that was available for each candidate gene promoter region. It did not evaluate the methylation status of CpG islands located in gene bodies or

the transcription start sites, nor did it evaluate the potential influence that DNA sequence variation (eg, SNPs) may have on the methylation status of neighboring CpGs.³⁴ While CpGs are most commonly found in gene promoters, CpGs may also be concentrated in gene bodies and island flanking shores and shelves, which are not captured in these targeted analyses. As differential methylation outside of promoter CpG islands have the potential to alter gene transcription, the unmeasured influence of allele-specific DNA methylation in these targets may underlie contributions to the preeclampsia phenotype. Another putative factor contributing to the association of DNA methylation in *ENG* and *TGF β 2* targets with preeclampsia reported in this study is the role of SNPs involving cytosine nucleotide bases underlying differential DNA methylation. Although methylation quantitative trait loci (meQTLs) have been identified for both *ENG* and *TGF β 2* within blood samples,³⁵ we were unable to investigate the impact of such meQTLs on methylation levels in our study due to lack of sequencing data. The lack of coverage, and knowledge of the underlying DNA sequence limits one's ability to fully interrogate the relationship between methylation levels and specific phenotypes of interest, and may have contributed to the null findings in this study. Future studies that capitalize on methods such as pyrosequencing or epigenome-wide methylation data collection, and also take methylation quantitative trait loci (meQTL) are thus needed.

Body mass index, smoking, and gestational age are other factors that can also affect methylation. Obesity increases the risk of developing preeclampsia and could influence methylation results.^{4,36} In this study, BMI was significantly higher in the cases included in the *TGF β 2* analysis, which could have influenced the results; however, we were unable control for BMI due to small sample size. Smoking has also been shown to influence methylation,^{37,38} with likely mechanisms associated with hypoxia, DNA damage, alteration in DNA-binding proteins and DNA methyltransferases.³⁴ Methylation status has also been shown to vary in the placenta with respect to gestational age.³⁹ Our small sample size prevented us from using multivariate modeling to control for this variability; however, our results demonstrated that the case and control groups were similar for these factors, with the exception that pre-pregnancy BMI was significantly higher in cases included in the *TGF β 2* analyses.

Strengths and limitations

There were several strengths and limitations associated with this study. Study strengths included matching on certain characteristics that could influence methylation, the use of a strict pregnancy outcome phenotype definition, and the ability to collect methylation data in duplicate, at a minimum, for each sample. The main study limitations included a small sample size, inability to collect methylation data on all samples, inability to conduct multivariate modeling, and inability to control for cell type heterogeneity.

The small sample size was in part due to our stringent and cautious data quality criteria. If we had relaxed our criteria to include samples that provided methylation data for at least one of the data runs, we would have additional 9 samples for the *ENG* analysis and an additional 2 samples for the *TGF β 2* analysis; however, we were committed to our conservative approach. As such, larger studies are needed to validate/replicate our findings. In addition, while our initial approach utilized 1:1 frequency matching to ensure that the case and control groups had similar distributions with respect to gestational age that the peripheral blood sample was collected (± 2 weeks), nulliparity, smoking status, and labor status during sample collection, the omission of samples from the analysis may have negated the matching. Although we did not detect any significant differences between the groups with respect to the matching variables, we may have not had adequate power to detect differences when they truly existed. Furthermore, the sample was restricted to self-reported White women who were nulliparous, which may limit the generalizability of findings to other self-reported races and multiparous women. The majority of the participants also delivered at term. As such, these findings may not represent early-onset preeclampsia, or other preeclampsia subtypes. Although the subtypes all result in the development of preeclampsia, the underlying pathophysiologic mechanisms likely differ among the subtypes. Because investigation of DNA methylation profiles may provide valuable insight into such biologic differences and may identify subtype specific biomarkers, future studies with large samples of preeclampsia subtypes are needed. Ultimately, such studies would improve our ability to more precisely identify and treat women. Lastly, we were unable to evaluate the association between methylation levels and *ENG* and *TGF β 2* genetic variation and/or gene expression. Although we had genetic variation data available for both candidate genes from a previous project, the sample size was too small to conduct genetic association tests. Because we did not have access to samples properly stored for evaluation of mRNA levels, we were unable to look at the association between methylation levels and gene expression levels in our study. Larger studies, with samples available for both DNA and mRNA analyses are therefore needed to assess whether or not there are relationships between DNA methylation levels and genetic variation and gene expression in these candidate genes.

In conclusion, we were unable to detect a significant association between DNA methylation in *ENG* pathway gene promoters and preeclampsia. The underlying mechanism(s) of preeclampsia is/are still not understood; however, previous research points to DNA methylation as a form of epigenetic regulation that affects gene expression and may represent a potential molecular mechanism that explains the relationship between the endoglin pathway and preeclampsia. As such, additional studies that include larger samples, investigate preeclampsia subtypes (eg, early-onset), and include a more comprehensive evaluation of *ENG* and *TGF β 2* DNA

methylation, and their relationships with DNA variation and mRNA expression, are needed to further investigate the association between *ENG* pathway methylation and preeclampsia. Exploring methylation profiles of other genes and gene pathways affecting vascular formation/function and placental implantation, and their association with preeclampsia development, should also be considered in future studies. Genes with significant variation in methylation have the potential to serve as blood-based biomarkers, differentiating preeclampsia from normotensive pregnancy and improving evidenced-based screening and treatment for these women.

Acknowledgements

The authors would like to thank Mrs. Sandra Deslouches, laboratory manager at the University of Pittsburgh School of Nursing Molecular Genetics Laboratory, for her continual assistance and mentoring of Ms. Rietze as she conducted her BSN-H thesis project.

Author Contributions

AHR: conceptualization; formal analysis; investigation; writing-original draft; writing-review and editing; project administration. YPC: conceptualization; resources; writing-review and editing; supervision; project administration; funding acquisition. DR: formal analysis; writing-review and editing; supervision. CMA: conceptualization; writing-review and editing. JMR: resources; writing-review and editing; funding acquisition. AJ: resources; writing-review and editing; funding acquisition. CAH: resources; writing-review and editing; funding acquisition. MJS: conceptualization; formal analysis; resources; writing-review and editing; supervision; project administration; funding acquisition.

REFERENCES

- Abalos E, Cuesta C, Grosso AL, Chou D, Say L. Global and regional estimates of preeclampsia and eclampsia: a systematic review. *Eur J Obstet Gynecol Reprod Biol.* 2013;170:1-7.
- Ananth CV, Keyes KM, Wapner RJ. Pre-eclampsia rates in the United States, 1980-2010: age-period-cohort analysis. *BMJ (Clinical research ed).* 2013;347:f6564.
- American College of Obstetricians and Gynecologists, Task force on hypertension in pregnancy. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task force on hypertension in pregnancy. *Obstet Gynecol.* 2013;122:1122-1131.
- Anderson CM, Schmella MJ. CE: preeclampsia: current approaches to nursing management. *Am J Nurs.* 2017;117:30-38.
- Chaiworapongsa T, Chaemsaitong P, Yeo L, Romero R. Pre-eclampsia part 1: current understanding of its pathophysiology. *Nat Rev Nephrol.* 2014;10:466-480.
- Roberts JM. The perplexing pregnancy disorder preeclampsia: what next? *Physiol Genomics.* 2018;50:459-467.
- Redman CW, Sargent IL, Staff AC. IFPA senior award lecture: making sense of pre-eclampsia - two placental causes of preeclampsia? *Placenta.* 2014;35:S20-S25.
- Gougas A, Letarte M. Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. *J Biol Chem.* 1990;265:8361-8364.
- Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C. Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-beta receptors I and II. *J Biol Chem.* 2002;277:29197-29209.
- St-Jacques S, Forte M, Lye SJ, Letarte M. Localization of endoglin, a transforming growth factor-beta binding protein, and of CD44 and integrins in placenta during the first trimester of pregnancy. *Biol Reprod.* 1994;51:405-413.
- Oujo B, Perez-Barriocanal F, Bernabeu C, Lopez-Novoa JM. Membrane and soluble forms of endoglin in preeclampsia. *Curr Mol Med.* 2013;13:1345-1357.
- Farina A, Zucchini C, Sekizawa A, et al. Performance of messenger RNAs circulating in maternal blood in the prediction of preeclampsia at 10-14 weeks. *Am J Obstet Gynecol.* 2010;203:575.e1-575.e7.
- Nishizawa H, Pryor-Koishi K, Kato T, Kowa H, Kurahashi H, Udagawa Y. Microarray analysis of differentially expressed fetal genes in placental tissue derived from early and late onset severe pre-eclampsia. *Placenta.* 2007;28:487-497.
- Purwosunu Y, Sekizawa A, Yoshimura S, et al. Expression of angiogenesis-related genes in the cellular component of the blood of preeclamptic women. *Reprod Sci.* 2009;16:857-864.
- Sekizawa A, Purwosunu Y, Farina A, et al. Prediction of pre-eclampsia by an analysis of placenta-derived cellular mRNA in the blood of pregnant women at 15-20 weeks of gestation. *BJOG.* 2010;117:557-564.
- Levine RJ, Lam C, Qian C, et al. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med.* 2006;355:992-1005.
- Rana S, Karumanchi SA, Levine RJ, et al. Sequential changes in antiangiogenic factors in early pregnancy and risk of developing preeclampsia. *Hypertension.* 2007;50:137-142.
- Venkatesha S, Toporsian M, Lam C, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med.* 2006;12:642-649.
- Bell MJ, Roberts JM, Founds SA, Jeyabalan A, Terhorst L, Conley YP. Variation in endoglin pathway genes is associated with preeclampsia: a case-control candidate gene association study. *BMC Pregnancy Childbirth.* 2013;13:82.
- Schmella MJ, Roberts JM, Conley YP, et al. Endoglin pathway genetic variation in preeclampsia: a validation study in Norwegian and Latina cohorts. *Hypertens Pregnancy.* 2018;12:144-149.
- Anderson CM, Ralph JL, Wright ML, Linggi B, Ohm JE. DNA methylation as a biomarker for preeclampsia. *Biol Res Nurs.* 2014;16:409-420.
- Ge J, Wang J, Zhang F, et al. Correlation between MTHFR gene methylation and pre-eclampsia, and its clinical significance. *Genet Mol Res.* 2015;14:8021-8028.
- White WM, Brost B, Sun Z, et al. Genome-wide methylation profiling demonstrates hypermethylation in maternal leukocyte DNA in preeclampsia compared to normotensive pregnancies. *Hypertens Pregnancy.* 2013;32:257-269.
- White WM, Sun Z, Borowski KS, et al. Preeclampsia/Eclampsia candidate genes show altered methylation in maternal leukocytes of preeclamptic women at the time of delivery. *Hypertens Pregnancy.* 2016;1-11.
- Ye W, Shen L, Xiong Y, Zhou Y, Gu H, Yang Z. Preeclampsia is associated with decreased methylation of the GNA12 promoter. *Ann Hum Genet.* 2016;80:7-10.
- Groen K, Lea RA, Maltby VE, Scott RJ, Lechner-Scott J. Letter to the editor: blood processing and sample storage have negligible effects on methylation. *Clin Epigenetics.* 2018;10:22.
- Li Y, Pan X, Roberts ML, et al. Stability of global methylation profiles of whole blood and extracted DNA under different storage durations and conditions. *Epigenomics.* 2018;10:797-811.
- Lind T, Godfrey KA, Otun H, Philips PR. Changes in serum uric acid concentrations during normal pregnancy. *Br J Obstet Gynaecol.* 1984;91:128-132.
- Qiagen. *Epitect Methyl II PCR Assay Handbook.* Germany: Qiagen; 2012.
- Fessele KL, Wright F. Primer in genetics and genomics, article 6: basics of epigenetic control. *Biol Res Nurs.* 2018;20:103-110.
- Abbassi-Ghanavati M, Greer LG, Cunningham FG. Pregnancy and laboratory studies: a reference table for clinicians. *Obstet Gynecol.* 2009;114:1326-1331.
- Gabbe SG, Niebyl JR, Simpson JL, et al. *Obstetrics: Normal and Abnormal Pregnancies.* 7th ed. Philadelphia, PA: Elsevier, Inc.; 2017.
- Sacks GP, Studena K, Sargent K, Redman CW. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. *Am J Obstet Gynecol.* 1998;179:80-86.
- Lee KW, Pausova Z. Cigarette smoking and DNA methylation. *Front Genet.* 2013;4:132.
- McClay JL, Shabalin AA, Dozmorov MG, et al. High density methylation QTL analysis in human blood via next-generation sequencing of the methylated genomic DNA fraction. *Genome Biol.* 2015;16:291.
- Durst JK, Tuuli MG, Stout MJ, Macones GA, Cahill AG. Degree of obesity at delivery and risk of preeclampsia with severe features. *Am J Obstet Gynecol.* 2016;214:651.e1-651.e5.
- Giannakou K, Evangelou E, Papatheodorou SI. Genetic and non-genetic risk factors for pre-eclampsia: umbrella review of systematic reviews and meta-analyses of observational studies. *Ultrasound Obstet Gynecol.* 2018;51:720-730.
- Zeilinger S, Kuhnel B, Klopp N, et al. Tobacco smoking leads to extensive genome-wide changes in DNA methylation. *PLoS One.* 2013;8:e63812.
- Leavey K, Wilson SL, Bainbridge SA, Robinson WP, Cox BJ. Epigenetic regulation of placental gene expression in transcriptional subtypes of preeclampsia. *Clin Epigenetics.* 2018;10:28.