BLOOD-BASED DNA METHYLATION LEVELS OF ENDOGLIN PATHWAY CANDIDATE GENES: DO THEY DIFFER IN WOMEN WITH AND WITHOUT CLINICALLY-OVERT PREECLAMPSIA?

by

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BLOOD-BASED DNA METHYLATION LEVELS OF ENDOGLIN PATHWAY CANDIDATE GENES: DO THEY DIFFER IN WOMEN WITH AND WITHOUT CLINICALLY-OVERT PREECLAMPSIA?

Allison Rietze, BSN-H

This observational pilot study was designed to test the hypothesis that blood-based DNA methylation levels of endoglin (*ENG*) and transforming growth factor beta receptor 2 (*TGFβR2*) gene promoter regions differ significantly when comparing women with clinically-overt preeclampsia to normotensive pregnant women matched on key variables. A 1:1 frequency matched case-control candidate gene design was used to evaluate *ENG* and *TGFβR2* gene promoter methylation levels. Methylation data were collected using the EpiTect Methyl II quantitative Polymerase Chain Reaction (PCR) Assay (Qiagen® Inc., Germantown, Maryland). The promoter region CpG islands evaluated included *ENG* (CpG Island 114642) and *TGFβR2* (CpG Island 110111). Genomic DNA was extracted from maternal peripheral white blood cells via protein precipitation. The sample included n=22 preeclampsia cases 1:1 frequency matched to n=22 normotensive controls on gestational age at sample collection (± 2 weeks), smoking status, and labor status at sample collection. All participants were Caucasian and nulliparous. Preeclampsia was diagnosed based on blood pressure, protein, and uric acid criteria. Parametric and nonparametric analyses were utilized to compare demographic and clinical characteristics between cases and controls. A non-parametric approach (Mann-Whitney U) was utilized to compare methylation levels for both candidate genes between cases and controls. Average methylation levels for both *ENG* (Cases [M±(SD)]= 6.54% ± 4.57; Controls= 4.81% ± 5.08; p=0.102) and *TGFβR2* (Cases= 1.5% ± 1.37; Controls= 1.7% ± 1.4; p= 0.695) promoter CpG islands did not significantly differ between cases and controls. The role that the ENG pathway

plays in preeclampsia pathogenesis is not fully understood. Evaluation of ENG pathway blood-based DNA methylation levels will better inform us of the potential role that *ENG* and *TGFβR2* DNA methylation plays in preeclampsia pathophysiology, including the maternal response to placental dysfunction. Although this study did not reveal detectable differences in blood-based DNA methylation levels of *ENG* and *TGFβR2* gene promoters during clinically-overt preeclampsia, additional epigenetic studies with larger sample sizes are needed to enhance our understanding of preeclampsia pathophysiology and to inform the development of prevention, detection (e.g. biomarkers), and treatment modalities that improve maternal and fetal health outcomes.

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PREFACE

I would like to acknowledge the following people for helping me with the production of this project for my Bachelor of Science in Nursing, Honors degree. Dr. Mandy Schmella for being an inspiring and involved mentor for the past two years, and always being available for guidance and encouragement. Dr. Yvette Conley for introducing me to the field of genomics research four years ago and for her continued support throughout this project. Dr. Dianxu Ren for his help with the analysis of my data and Dr. Cindy Anderson for participating as a member of my defense committee. Drs. Carl Hubel, Arun Jeyabalan, and James Roberts for their assistance and support. Dr. David Hornyak for being a helpful resource at the Honors College. Sandra Deslouches for her constant help in the lab and encouraging words when things did not always go as planned. And my family for their motivation, support, and for driving all this way for my defense.

1.0 BACKGROUND AND SIGNIFICANCE

Preeclampsia is a multi-system, pregnancy-specific disorder that affects approximately three to five percent of pregnancies (Ananth, Keyes, & Wapner, 2013; Abalos, Grosso, Chou & Say, 2013) and is significantly associated with poor health outcomes for both the mother and the fetus/infant (Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy, 2013). Maternal complications and signs/symptoms of preeclampsia include severe headaches, changes in vision, coagulation problems related to thrombocytopenia, respiratory dysfunction, renal and hepatic failure, and heart disease. Moreover, preeclampsia can lead to premature birth, placental abruption, intrauterine growth restriction, low birthweight, or death of the mother and/or baby (Abalos et al., 2014; Creanga et al., 2015; Duley, 2009; Goldenberg, Culhane, Iams, & Romero, 2008; Kuklina, Ayala, & Callaghan, 2009; Mor et al., 2016). In the United States, three out of 20 premature births can be attributed to unmanaged preeclampsia (March of Dimes, 2016). Furthermore, the mother and child are also at increased risk for future cardiovascular dysfunction remote from pregnancy (Brown et al., 2013; Davis et al., 2012; Timpka et al., 2016; Wu et al., 2017).

Preeclampsia is characterized as new onset hypertension accompanied by proteinuria, or signs of multisystem involvement in the absence of proteinuria, after 20 weeks' gestation in a previously normotensive woman (Hypertension in pregnancy. Report of the American College of

Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy, 2013). Preeclampsia is seen more frequently in women who are nulliparous, pregnant with multiples, or are much younger or older than the ideal age for pregnancy (Anderson, Ralph, Wright, Linggi, & Ohm, 2014). Although preeclampsia is a significant cause of maternal and infant morbidity and mortality, the pathophysiology is not completely understood, creating a barrier to predicting and preventing its development (Chaiworapongsa, Chaemsaithong, Yeo, & Romero, 2014). Furthermore, the only known cure for preeclampsia is delivery of the placenta. In order to screen for and prevent preeclampsia, the underlying disorder must be better understood, and in turn, efforts can be directed towards the development of preventative, screening, and treatment modalities that aim to improve the health outcomes of women and their newborns (Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy, 2013).

Although it is believed that the development of preeclampsia stems from abnormalities in spiral artery transformation, placental implantation, and an unfavorable maternal response, the causes of these abnormalities have not been fully elucidated (Chaiworapongsa et al., 2014). This lack of knowledge has hampered the ability to prevent preeclampsia, detect preeclampsia before it becomes clinically overt, and treat preeclampsia. Studies looking at gene pathways that are involved with angiogenesis in the nervous system, cardiovascular system, and others have been completed, but a biomarker of preeclampsia has not been identified (Anderson et al., 2014; Anderson & Schmella, 2017; Ge et al., 2015; Roberts, 2018; White et al., 2013; White et al., 2016; Ye et al., 2016). ENG and TGFβR2 have been recognized as proteins present on proliferating vascular endothelial cells and placental syncytiotrophoblasts that play a role in the development of vascular disease, including preeclampsia (ten Dijke, Goumans, & Pardali, 2008).

ENG is a receptor protein encoded by the endoglin gene that is expressed on proliferating vascular endothelial cells and trophoblast cells of the placenta (Caniggia et al., 1997; ten Dijke, Goumans, & Pardali, 2008). ENG is a co-receptor of the transforming growth factor beta (TGFβ) signaling system that is involved in cell proliferation, differentiation, motility, and apoptosis (ten Dijke, Goumans, & Pardali, 2008). Along this pathway, ENG regulates vascular tone and angiogenesis and regulates placental implantation/spiral artery remodeling (Caniggia et al., 1997; Jerkic et al., 2004; Toporsian et al., 2005).

TGFβR2 is another receptor protein that is a member of the TGFβ receptor signaling pathway that is expressed on the trophoblast of the placenta and vascular endothelial cells (Caniggia et al., 1997; Venkatesha et al., 2006). TGFβR2 is involved in vascular morphogenesis and in preventing endothelial cells from growing in an uncontrolled manner, whose signals are regulated and modified by ENG (Caniggia et al., 1997; Genetics Home Reference, 2018; ten Dijke, Goumans, & Pardali, 2008). In preeclampsia, vascular homeostasis is disrupted and placental implantation/spiral artery remodeling is often inadequate, which provides biologic plausibility for the study of ENG and TGFβR2.

There are multiple lines of evidence that support a role for the endoglin pathway in the development of preeclampsia. First, the soluble form of endoglin (sENG), which is thought to be generated from the cleavage of membrane-bound ENG from the placenta and released into the maternal circulation, is elevated in the maternal circulation of women before clinically-overt preeclampsia (Levine et al., 2006; Rana et al., 2007). When present in the maternal circulation, it is thought that sENG interferes with downstream signaling along the TGFβ pathway (Venkatesha et al., 2006). Second, *ENG* expression (mRNA) has been found to be elevated in the cellular component of maternal blood during all three trimesters of pregnancy, as well as in

the placenta obtained via cesarean section before labor onset (Farina et al., 2010; Nishizawa et al., 2007; Purwosunu et al., 2009; Sekizawa et al., 2010). Third, maternal genetic variation in *ENG* and *TGFβR2* have been implicated in susceptibility to/protection from preeclampsia through genetic association studies; however, mechanisms that may explain these associations have not been defined (Bell et al., 2013).

DNA methylation is a form of epigenetic regulation that can greatly affect gene expression and is a potential molecular mechanism that may explain the role of the endoglin pathway, including the genetic association between the ENG pathway, and susceptibility to/protection from preeclampsia. In a small pilot study, differences in blood-based, genome-wide DNA methylation profiles were detected during the first trimester in women who developed preeclampsia compared to women who had uncomplicated pregnancies, however, these differences were not detected in *ENG* or *TGFβR2* (Anderson et al., 2014). Several others studies have also evaluated DNA methylation profiles in maternal peripheral white blood cells using both candidate gene and genome-wide approaches. The results of the studies by Anderson et al. (2014), Ge et al. (2015), White et al. (2013), White et al. (2016), and Ye et al. (2016), have generally 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, when looking at DNA methylation in maternal peripheral blood as it relates to the development of preeclampsia, there is a lack of research specifically looking at methylation levels of *ENG* and *TGFβR2*.

2.0 PURPOSE

The purpose of this observational pilot study was to determine 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. Therefore, the specific aim of this study was the following:

Specific Aim: Compare maternal blood-based DNA methylation levels of the *ENG* and *TGFβR2* gene promoters in women with clinically-overt preeclampsia to that in normotensive women. DNA extracted from maternal peripheral blood will be used to detect methylation-levels of *ENG* and *TGFβR2* gene promoters in cases (clinically-overt preeclampsia) and controls (normotensive pregnancies).

3.0 METHODS

3.1 DESIGN

A targeted, candidate gene methylation approach was used to interrogate and compare methylation status of *ENG* and *TGFβR2* gene promoter CpG islands. Samples and demographic/clinical data were received from the Prenatal Exposures and Preeclampsia (PEPP) study conducted at Magee-Womens Hospital of the University of Pittsburgh Medical Center, in a de-identified manner. Participants were 1:1 frequency matched on gestational age that the sample was collected (+/- 2 weeks), nulliparity, smoking status, and labor status when the sample was collected, as these characteristics could impact methylation. *ENG* and *TGFβR2* promoter DNA methylation levels in the blood of women with clinically-overt preeclampsia to women with uncomplicated pregnancies were compared.

3.2 PARTICIPANTS

This study was reviewed and approved by the University of Pittsburgh Human Research Protection Office. Participants included in this study originally participated in the PEPP study, cohorts 1 and 2. The purpose of PEPP was to investigate factors related to the development of preeclampsia. Participants recruited for PEPP 1 and 2 were pregnant women between the ages of

14 and 44 years. They were recruited either at 20 weeks' gestation, or earlier, and were followed through delivery or they were recruited cross-sectionally during labor due to suspected preeclampsia. Women were excluded from 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 participants included in this thesis project were self-reported Caucasian, including n=22 preeclampsia cases who were 1:1 frequency matched to n=22 normotensive controls on gestational age that the sample was collected (± 2 weeks), nulliparity, smoking status, and labor status when the sample was collected. Cases were defined as having clinically overt preeclampsia when the 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 four most recent blood pressures taken in the hospital prior to therapeutic intervention; (2) proteinuria ≥ 300mg/24 hours, ≥ 0.3 protein/creatinine ratio, ≥ 2+ on a random urine specimen, or ≥ 1+ on a catheterized urine specimen; and (3) hyperuricemia with serum uric acid concentration ≥ 1 standard deviation from normal for gestational age. Controls were defined as women who remained normotensive throughout their entire pregnancy, without developing proteinuria and delivered healthy term babies.

3.3 DNA EXTRACTION AND QUANTIFICATION

Genomic DNA was extracted via protein precipitation from maternal peripheral blood samples that were collected during the third trimester. De-identified DNA aliquots were provided by

PEPP investigators to the principal investigator and her thesis advisor. Genomic DNA was quantified at the University of Pittsburgh Genomics Research Core.

3.4 METHYLATION DATA COLLECTION

Methylation data were collected using EpiTect Methyl II PCR Assays (Qiagen® Inc., Germantown, Maryland) after DNA quantification, and all participant samples were run in duplicate for both the *ENG* and *TGFβR2* assays. The values were reviewed from these two rounds of data collection. If methylation data for a participant sample were generated for both rounds of data collection, and their methylation values were concordant (|Run 1 value (% methylated) – Run 2 value (% methylated)| ≤ 15% for both *ENG* and *TGFβR2*), the two methylation values were averaged. In the event that (1) methylation data for a participant sample were only generated for one of the two rounds of data collection or (2) methylation data generated for the first two rounds of data collection for a participant sample were discordant (|Run 1 value (% methylated) – Run 2 value (% methylated)| ≥ 15% for *ENG* and/or *TGFβR2*), the participant sample underwent a third round of data collection. Samples that failed to generate data for the first two rounds of data collection were not included in the third round of data collection, and they were omitted from analysis. Only samples that had two concordant values were included in the final analysis, with the average of these two values used for analysis (Figure 1).

To begin methylation data collection, a reaction mix was first prepared using a volume of the samples derived from the DNA quantification (to achieve a DNA concentration of >4µg/mL), 5X Restriction Digestion Buffer, and RNase-/DNase-free water, for a total volume of

60µl per sample. The reaction mix was prepared in 0.5mL microcentrifuge tubes and stored at -20˚C. A restriction digestion was then carried out using the reaction mixes. The plates were set up so that each sample was combined separately with no enzyme in one well, a methylation-sensitive restriction enzyme (MSRE) in another well, a methylation-dependent restriction enzyme (MDRE) in a third well, and a combination of MSRE and MDRE in the last well. RNase-/DNase-free water was then added to the entire plate for a total volume of 15µl per well. The sample plate was digested in a thermal cycler at 37˚C for 6 hours or overnight, and then at 65˚C for 20 minutes. A polymerase chain reaction (PCR) was then set up using the digested product from the previous step and combined with PCR Master Mix (SYBR Green) (Applied Biosystems™, Foster City, California), PCR Primer Mix, and more RNase-/DNase-free water. Each sample was run with two different primer mixes: EpiTect Methyl II PCR Primer Assay for Human ENG (CpG Island 114642; Catalogue number: EPHS114642-1A; Qiagen® Inc., Germantown, Maryland) in one plate, and EpiTect Methyl II PCR Primer Assay for Human TGFβR2 (CpG Island 110111; Catalogue number: EPHS110111-1A; Qiagen® Inc., Germantown, Maryland) in another plate. The PCR was run in the 7000 Sequence Detection System (Applied Biosystem™, Foster City, California) using an absolute quantification (standard curve) data collection setting. The EpiTect Methyl II Assay Handbook indicates the following: the product digested with no enzyme represents total input DNA for PCR detection; the MSRE digests unmethylated and partially methylated DNA and hypermethylated DNA is detected with PCR; the MDRE digests methylated DNA and the unmethylated DNA is detected with PCR; and the MSRE-MDRE combination should digest all DNA molecules (Qiagen, 2012). Tables 1-5 detail each step of the methylation data collection protocol.

Table 1. Methylation Data Collection Step 1: Preparing Reaction Mix

DNA (125 ng)	*Volume based on DNA concentration of sample*[a]
5X Restriction Digestion Buffer	13µl
RNase-/DNase-free water	*Volume dependent on amount of DNA*
Total Volume:	60µl

*Notes.*Table adapted from EpiTect Methyl II PCR Assay Handbook (Qiagen, 2012). [a]The recommended DNA concentration is > 4µg/mL (Qiagen, 2012).

Table 2. Methylation Data Collection Step 2: Restriction Digestion

	M_0 (A)	**M_S (C)**	**M_D (E)**	**M_{S+D} (G)**
Reaction Mix (Step 1)[a]	14µl	14µl	14µl	14µl
MSRE	X	0.5µl	X	0.5µl
MDRE	X	X	0.5µ1	0.5µ1
RNase-/DNase-free water	1µl	0.5µl	0.5µ1	X
Total Volume:	15µl	15µl	15µl	15µl

Notes. Table adapted from EpiTect Methyl II PCR Assay Handbook (Qiagen, 2012**)**. [a]Mixture from Methylation Data Collection Step 1. M_0(A)= No restriction enzyme, plate row A. M_S(C)= Methylation sensitive enzyme, plate row C. M_D(E)= Methylation dependent enzyme, plate row E. M_{S+D}(G)= Methylation sensitive and dependent enzymes, plate row G. MSRE= Methylation-Sensitive Restriction Enzyme. MDRE= Methylation-Dependent Restriction Enzyme.

Table 3. Methylation Data Collection Step 3: Polymerase Chain Reaction (PCR) Setup

	M_0 (A)	**M_S (C)**	**M_D (E)**	**M_{S+D} (G)**
PCR Master Mix (SYBR Green)	12.5µl	12.5µl	12.5µl	12.5µl
PCR Primer Mix (ENG or TGFβR2)	1µl	1µl	1µl	1µl
M_0 Digest	5µl	X	X	X
M_S Digest	X	5µl	X	X
M_D Digest	X	X	5µl	X
M_{S+D} Digest	X	X	X	5µl
RNase/DNase-free water	6.5µl	6.5µl	6.5µl	6.5µl
Total Volume:	25µl	25µl	25µl	25µl

Notes. Table adapted from EpiTect Methyl II PCR Assay Handbook (Qiagen, 2012). M_0(A)= No restriction enzyme, plate row A. M_S(C)= Methylation sensitive enzyme, plate row C. M_D(E)= Methylation dependent enzyme, plate row E. M_{S+D}(G)= Methylation sensitive and dependent enzymes, plate row G. M_0 Digest= Product from Methylation Data Collection Step 2, row A. M_S Digest= Product from Methylation Data Collection Step 2, row C. M_D Digest= Product from Methylation Data Collection Step 2, row E. M_{S+D} Digest= Product from Methylation Data Collection Step 2, row G.

Table 4. Thermal Cycler Protocol: Incubation for Restriction Digestion

Stage 1	37˚C	6 hours or overnight
Stage 2	65˚C	20 minutes

Notes. Table adapted from EpiTect Methyl II PCR Assay Handbook (Qiagen, 2012).

Table 5. PCR Cycling Protocol (7000 Sequence Detection System)

Stage 1	Step 1	95˚C	10min	1 cycle
Stage 2	Step 1	99˚C	30sec	3 cycles
	Step 2	72˚C	1min	
Stage 3	Step 1	97˚C	15sec	40 cycles
	Step 2	72˚C	1min[a]	

Notes. Table adapted from EpiTect Methyl II PCR Assay Handbook (Qiagen, 2012). [a]Data collection occurs during Step 2 of Stage 3.

3.5 RATIONALE FOR FINAL SAMPLE SIZE

Throughout the study, several participants were eliminated from the analysis for various reasons. Initially, two participants were omitted, as they were not nulliparous. A third participant was later omitted due to a misclassification of pregnancy outcome. These omissions resulted in a sample size of N=41, with n=20 cases and n=21 controls. Methylation data was collected for all samples in duplicate, then any samples that had two disparate values or only one value from the first two runs of data collection, underwent a third round of data collection. The data collected were reviewed, and any samples that either (1) failed both of the first two data collection rounds, (2) generated data for only one round of data collection, (3) or generated discordant data values that differed by more than 15%, were omitted from the final analysis. The final analysis included N= 35 participants (18 cases, 17 controls) for demographic data, N= 20 participants (9 cases, 11 controls) for *ENG* methylation data, and N=28 participants (15 cases, 13 controls) for *TGFβR2* methylation data.

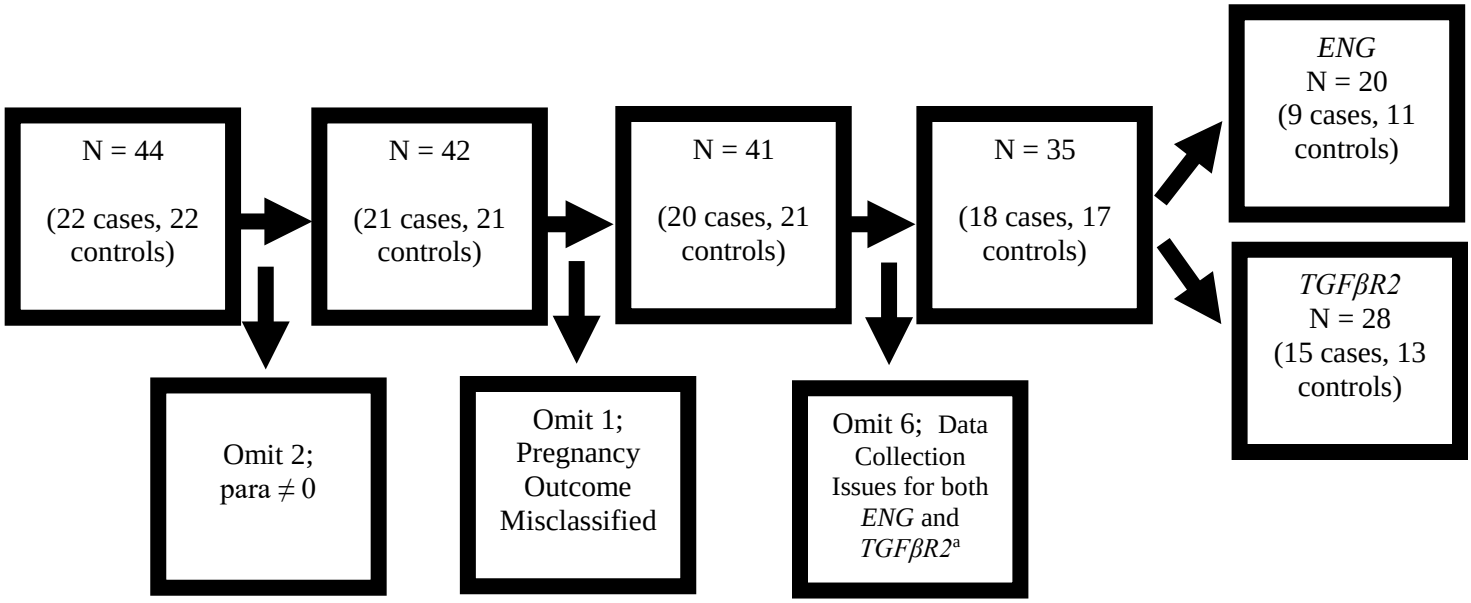

Figure 1. Sample Size Flowchart

Notes. [a]Participants were omitted for one of the following reasons: Could not obtain methylation values for two rounds of data collection, could never obtain values, or the two values were disparate, when run with both *ENG* and *TGFβR2*.

3.6 STATISTICAL ANALYSIS

The data were analyzed using IBM's SPSS Statistics Version 24 software (IBM Corp., Armonk, New York). Our main outcome of average methylation levels was evaluated using bivariate analysis, comparing values between cases and controls represented as percentages. Continuous demographic/clinical variables were analyzed either using the parametric Independent Samples t-test or the non-parametric Mann-Whitney U Test, depending on whether the variables were normally distributed. Categorical demographic/clinical variables were analyzed using the Pearson Chi-Square test. We were unable to control for potential confounders in multivariate analysis due to the small sample size.

4.0 RESULTS

4.1 DEMOGRAPHIC/CLINICAL CHARACTERISTICS

Several characteristics were found to be similar between participants with clinically-overt preeclampsia and normotensive control participants (Table 6). All participants were self-reported Caucasian and nulliparous. Gestational age at sample collection was similar between groups, which was expected given that the cases and controls were 1:1 frequency matched on gestational age at sample collection, with the majority of samples not being collected in labor. Furthermore, cases and controls were similar with respect to maternal age, gestational age at delivery (Cases (*M(SD))*: 37.56(2.95); Controls *(M(SD))*: 39.4(1.15)), and smoking status. The majority of participants were non-smokers (Cases: 55.6%; Controls: 58.8%). Average systolic blood pressure before 20 weeks' gestation was also similar between cases and controls. Additionally, frequency matching remained the same for both subsets that were analyzed for *ENG* and *TGFβR2* methylation after samples were omitted (data not shown).

Participants with clinically-overt preeclampsia and normotensive control participants also differed in regard to several characteristics (Table 6). Average diastolic blood pressure before 20 weeks' gestation was significantly higher in cases compared to controls (although the blood pressure ranges were within normal limits). Both average systolic and diastolic blood pressure measurements during labor were significantly higher in cases compared to controls—an expected

finding given the research definition and clinical presentation of preeclampsia. Average plasma sENG levels were significantly higher in cases (Cases [M±(SD)]= 31.5ng/mL ± 21.03; Controls= 10.5ng/mL ± 4.86), which demonstrates a similar trend to the study completed by Levine et al. (2006). Pre-pregnancy body mass index (BMI) was found to be significantly different between groups, with cases having higher BMIs than controls, which was expected given the increased risk of preeclampsia that is associated with obesity (Anderson & Schmella, 2017; Durst, Tuuli, Stout, Macones, & Cahill, 2016).

Table 6. Demographic/Clinical Characteristics

Variables	Cases (n=18)	Controls (n=17)	p-Value
Maternal age, years *(M(SD))*	29.5(6.08)	27.76(6.02)	0.402[a]
Gestational age at delivery, weeks *(M(SD))*	37.56(2.95)	39.4(1.15)	0.057[b]
Gestational age at sample collection, weeks *(M(SD))*	37.45(2.99)	37.24(3.26)	0.791[b]
Average SBP <20wks, mmHg *(M(SD))*	115.94(9.4)	112.65(7.91)	0.277[a]
Average DBP <20wks, mmHg *(M(SD))*	72.41(4.93)	67.65(5.71)	0.014[a]
Average SBP in labor, mmHg *(M(SD))*	152.11(10.48)	118.65(11.7)	<0.001[a]
Average DPB in labor, mmHg *(M(SD))*	90.22(6.84)	70.24(8.45)	<0.001[a]
Pre-pregnancy BMI, kg/m^2 *(M(SD))*	29.32(6.71)	24.5(6.54)	0.039[a]
Average plasma sENG, ng/mL *(M(SD))*	31.5(21.03)	10.5(4.86)	<0.001[b]
Caucasian (n(%))	18(100%)	17(100%)	N/A
Nulliparous (n(%))	18(100%)	17(100%)	N/A
Smoking Status (n(%)) **No**	10(55.6%)	10(58.8%)	0.845[c]
Sample Collected in Labor (n(%)) **No**	12(66.7%)	11(64.7%)	0.903[c]

Note. *M(SD)*= mean (standard deviation). SBP= systolic blood pressure. DBP= diastolic blood pressure. mmHg= millimeters of mercury. BMI=body mass index. [a]Independent samples t-test. [b]Mann-Whitney U test. [c]Pearson Chi-Square test.

4.2 *ENG* AND *TGFBR2* METHYLATION RESULTS

Participants with clinically-overt preeclampsia were found to have higher levels of DNA methylation with respect to the CpG island promoter region of *ENG* compared to controls, but this difference was not statistically significant (Table 7). The CpG island in the promoter region of *TGFβR2* was also not differentially methylated between cases and controls (Table 7). Methylation results for both *ENG* and *TGFβR2* were not normally distributed, which is why a non-parametric analytic approach was used. For *ENG*, the minimum percent methylated value among cases was 2.24%, with a maximum percent methylated value of 17.46%; among controls, the minimum percent methylated value was 1.65% and the maximum percent methylated value was 19.3%. For *TGFβR2*, the minimum percent methylated value among cases was 0.01% and the maximum percent methylated value was 5.24%; among controls, the minimum percent methylated value was 0.19% and the maximum percent methylated value was 4.22%.

Table 7. *ENG & TGFβR2* Methylation Results

		Cases(n=9)	Controls(n=11)	p-value
ENG,	***M(SD)***	6.54(4.57)	4.81(5.08)	
% Methylated				0.102[a]
	Median(IQR)	5.2(3.57, 7.86)	2.72(2.38, 6.04)	
		Cases(n=15)	**Controls(n=13)**	**p-value**
TGFβR2,	***M(SD)***	1.5(1.37)	1.7(1.4)	
% Methylated				0.695[a]
	Median(IQR)	1.5(0.2, 2.12)	1.51(0.48, 2.64)	

Note. M(SD)= mean (standard deviation). *Median(IQR)*= median (inter-quartile range). [a]Mann-Whitney U test.

5.0 DISCUSSION

This observational, pilot 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 explaining the pathway's role in preeclampsia. Neither of the CpG islands within the promoter regions of *ENG* and *TGFβR2* were found to be differentially methylated between women with clinically-overt preeclampsia and women with normotensive, uncomplicated pregnancies. These findings could indicate that there is no relationship between methylation of these promoters and the presence or absence of clinically-overt preeclampsia; however, this pilot study may have been underpowered to detect statistically significant differences when they truly existed. As such, additional studies with larger sample sizes are needed to further investigate the association between ENG pathway methylation and preeclampsia.

When designing epigenetic studies, there are important factors to consider that can impact the collection and interpretation of methylation data: (1) tissue type, (2) cell type heterogeneity, and (3) 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. Additionally, 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 pilot study, includes not correcting for cell type heterogeneity (Anderson et al., 2014; White et al., 2013; White et al., 2016). 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. As such, not controlling for this could impact the interpretation/validity of one's results (Abbassi-Ghanavati, Greer, & Cunningham, 2009; Gabbe et al., 2017; Sacks, Studena, Sargent, & Redman, 1997). It will be important to look further into the role of ENG in white blood cells in the setting of the maternal circulation.

BMI, smoking, and gestational age are other factors that can also impact methylation. The difference in BMI between cases and controls was significant, with cases having higher pre-pregnancy BMIs than controls. Obesity increases the risk of developing preeclampsia and could impact methylation results (Anderson & Schmella, 2017; Durst et al., 2016). Smoking has also been shown to impact methylation (Giannakou, Evangelou, & Papatheodorou, 2017; Zeilinger, 2013). Methylation status has also been shown to vary in the placenta with respect to gestational age (Leavey, et al., 2018). However, due to the small sample size, we were unable to use multivariate modeling to control for this variability.

5.1 STRENGTHS AND LIMITATIONS

There were several strengths and limitations associated with this study. Matching on certain characteristics that could impact methylation (gestational age at sample collection, nulliparity, smoking status, and labor status at sample collection) was one strength of this study. The cases of this study were labeled as such based on a strict phenotype definition of preeclampsia, while the controls were also based on a strict phenotype definition. Furthermore, a major strength was our ability to collect methylation data in duplicate for each sample, and then a third time for any samples that needed to make-up for any failed runs or disparate results.

The small sample size was a main limitation of this study. We started with 22 cases and 22 controls, but we ended up with 18 cases and 17 controls due to a discrepancy in demographic data or failure to collect consistent methylation values. The small sample size further prohibited the use of multivariate modeling in which the effects of potential confounders on methylation could be accounted for. Additionally, the sample was all self-reported Caucasian, limiting the generalizability of findings, and we were unable to control for cell type heterogeneity, which could impact the validity of our results.

5.2 CONCLUSION AND FUTURE DIRECTIONS

This observational, pilot study was unable to detect a significant association between DNA methylation 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 affecting gene expression and a potential molecular

mechanism explaining this association. Future studies that include a larger sample size, control for cell type heterogeneity, and control for potential confounders, are needed to validate previous findings. 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.

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