GENOMICS OF ENDOGLIN PATHWAY IN PREECLAMPSIA

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Preeclampsia is a pregnancy disorder that greatly impacts maternal and fetal/neonatal health and wellbeing. This case-control candidate gene association study investigated endoglin pathway genetic variation and its association with preeclampsia. Tagging single nucleotide polymorphisms (tSNPs) in *ENG*, *TGFβ1*, *TGFβR1*, *ALK1*, and *TGFβR2* were genotyped with iPLEX® and TaqMan® in maternal/fetal dyads. The Prenatal Exposures and Preeclampsia Prevention study provided maternal DNA extracted from peripherally collected white blood cell pellets, along with umbilical cord serum we used for fetal DNA extraction. Data on 355 white (181 cases/174 controls) and 60 black (30 cases/30 controls) women matched on ancestry, age, and parity were analyzed. Separate subgroup allele/genotype/haplotype tests were conducted with Chi-square or Fisher's exact tests. Binary logistic regression provided odds ratios for tSNPs with significant genotype tests. Analysis of maternal/fetal dyads was not conducted, because unlike the maternal samples, the fetal samples did not provide a quality template suitable for iPLEX® data collection. In white women, variation in *ENG* (rs11792480, rs10121110) and *TGFβR2* (rs6550005) was associated with preeclampsia. Allelic frequency distributions in rs11792480, rs10121110, and rs6550005 were significantly different among cases and controls while genotype distributions of rs10121110 and rs6550005 were further associated with preeclampsia (p-values < .05). For rs10121110, women with the AA genotype were 2.290 times more likely to develop preeclampsia compared to the GG genotype (99% CI [1.022, 5.133], p =

.008). *ENG* haplotype TACGA, which contains rs11792480 and rs10121110 risk alleles, was also over-represented in cases (p = .022). In black women, variation in *TGFβ1* (rs4803455, rs4803457), *TGFβR1* (rs10739778), and *TGFβR2* (rs6550005, rs1346907, rs877572) was associated with preeclampsia. Allelic frequency distributions in rs10739778, rs6550005, rs1346907, and rs877572 were significantly different among cases and controls while genotype distributions of rs10739778, rs4803455, and rs4803457 were further associated with preeclampsia (p-values < .05). For rs4803457, women with the CT genotype were 7.437 more times likely to develop preeclampsia compared to the CC genotype (99% CI [1.192, 46.408], p = .005). These results demonstrate that variation in *ENG* pathway genes is associated with preeclampsia, with different genes from the same pathway contributing to preeclampsia in white compared to black women.

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1.0 PROPOSAL INTRODUCTION

Preeclampsia represents a hypertensive, multi-system pregnancy disorder that is significantly associated with maternal and fetal/neonatal morbidity and mortality (National Heart, Lung, and Blood Institute [NHLBI] National High Blood Pressure Education Program, 2000). In preeclampsia, shallow trophoblast invasion, insufficient remodeling of the maternal vessels perfusing the placenta (Brosens, Robertson, & Dixon, 1967; Brosens, Roberston, & Dixon, 1972; Gerretsen, Huisjes, & Elema, 1981; Khong, De Wolf, Robertson, & Brosens, 1981; Zhou, Damsky, Chiu, Roberts, & Fisher, 1993; Zhou, Damsky, & Fisher, 1997), and alterations in vascular endothelial function (American College of Obstetricians and Gynecologists [ACOG], 2002) have been well documented. However, the underlying processes responsible for these aberrations have not been fully elucidated and the only curative treatment remains delivery. As a result, it is imperative that scientific research continues to investigate the underlying mechanisms contributing to preeclampsia development. Such research can be utilized to design interventions (prevention, detection, treatment) that improve health outcomes of mothers and babies.

Gene expression studies have identified endoglin (*ENG*) as a factor potentially involved in preeclampsia development (Farina et al., 2008; Kim et al., 2009; Levine et al., 2006; Lim et al., 2009; Masuyama, Nakatsukasa, Takamoto, & Hiramatsu, 2007; Nishizawa et al., 2007; Rana et al., 2007; Robinson & Johnson, 2007; Romero et al., 2008; Salahuddin et al., 2007; Sitras et

al., 2009; Stepan et al., 2008; Toft et al., 2008). ENG is a co-receptor of the TGF-β family (Cheifetz et al., 1992) that is involved in regulation of trophoblast invasion (Caniggia, Taylor, Ritchie, Lye, & Letarte, 1997) and vascular endothelial function (Jerkic et al., 2004; Toporsian et al., 2005), two processes disrupted in preeclampsia (Roberts & Hubel, 2009). Given ENG's biological plausability in preeclampsia development, further research investigating its potential role in preelcampsia is warranted and may improve our understanding of preeclampsia.

1.1 PURPOSE AND SPECIFIC AIMS

Because investigation of the *ENG* pathway at the molecular level is needed, this pathway specific, candidate gene, nested, case-control research study will:

1. Investigate variation in maternal genes involved in the *ENG* pathway for impact on development of preeclampsia
2. Explore variation in maternal/fetal dyad genes involved in the *ENG* pathway for impact on development of preeclampsia

The candidate's broad, long term objective of her program of research is to improve the scientific/healthcare community's knowledge of preeclampsia pathophysiology in an effort to reduce the overall morbidity and mortality associated with preeclampsia. Study findings from this proposed disseration research will assist in the achievement of this long term objective. Results from this proposed research study may explain variability in susceptibility to preeclampsia, increase knowledge of preeclampsia pathophysiology, and assist in the development of interventions aimed at the prevention, detection, and treatment of preeclampsia.

1.2 BACKGROUND AND SIGNIFICANCE

Preeclampsia (PE), which is characterized by the development of hypertension and proteinuria after 20 weeks' gestation in a previously normotensive woman, significantly impacts maternal and fetal/neonatal morbidity/mortality (ACOG, 2002; NHLBI National High Blood Pressure Education Program, 2000). Complicating 3-5% of pregnancies (Roberts & Cooper, 2001), PE is estimated to cost the USA $7 billion annually (Preeclampsia Foundation, 2000-2010). Potential short term maternal complications include placental abruption, disseminated intravascular coagulation, cerebral hemorrhage, renal/hepatic failure, and death (NHLBI National High Blood Pressure Education Program, 2000) while short term fetal/neonatal complications include IUGR, low amniotic fluid levels (ACOG), premature birth, low birth weight, and stillbirth (NHLBI, n.d.). In addition, the development of PE can remotely impact the health of the mother and her infant. Long term maternal complications include elevated cardiac morbidity (Berends et al., 2008; Brown et al., 2006; Forest et al., 2005) and mortality (Arnadottir, Geirsson, Arngrimsson, Jonsdottir, & Olafsson, 2005; Irgens, Reisaeter, Irgens, & Lie, 2001). For premature infants, long term complications include increased risk of cerebral palsy, mental retardation, chronic lung disease, and vision/hearing problems (March of Dimes, 2007). Despite the range of short and long term complications, efforts to prevent these sequelae are impeded by the unknown etiology of PE. As a result, interventions are symptom driven and delivery of the placenta is the only known cure (Norwitz & Repke, 2010).

1.2.1 Rationale for genetic research

PE is to some extent based on hereditability (Nilsson, Salonen Ros, Cnattingius, & Lichtenstein, 2004; Skjaerven et al., 2005), which provides justification for candidate gene studies. In daughters born to mothers diagnosed with PE during pregnancy, research has shown that these daughters had 2 times the odds of developing PE compared with other women (OR =2.2; 95% CI [2.0-2.4]) (Skjaerven et al.). Research has also shown that when comparing a woman whose full sister did not develop PE during pregnancy, a woman whose full sister developed PE during pregnancy had 3 times the odds of developing PE during her pregnancy (OR=3.3; 95% CI [3.0-3.6]) (Nilsson et al.). Moreover, in sons of mothers diagnosed with PE during their pregnancy, the odds of fathering a PE pregnancy in the first pregnancy with their partner has been shown to be increased (OR=1.5; 95% CI [1.3-1.7]) (Skjaerven et al.). Thus, evidence suggests that PE may be partly attributed to inheritance.

1.2.2 Biological plausibility of endoglin (ENG)

ENG is a membrane bound glycoprotein and co-receptor of transforming growth factor beta (TGFβ) (Cheifetz et al., 1992) that modulates TGFβ signal transduction via interaction with TGFβ type 1 and 2 receptors (Guerrero-Esteo, Sánchez-Elsner, Letamendia, & Bernabéu, 2002). TGFβ ligands first bind to a receptor complex comprised of a type I (e.g., ALK1 and ALK5) and type II receptor (TGFβR2). Once this complex is activated, the cellular signal is transferred to the mothers against DPP homologs (SMAD) proteins. The SMAD proteins transmit TGFβ signals from the cell-surface receptors to the nucleus and, as nuclear effectors, are involved in

the regulation of target gene transcription (Lebrin, Deckers, Bertolino, & ten Dijke, 2005; Online Mendelian Inheritance in Man [OMIM], 2010; ten Dijke, Goumans, & Pardali, 2008).

ENG is expressed on syncytiotrophoblasts/transitioning cytotrophoblast cells of the placenta (St-Jacques, Forte, Lye, &Letarte, 1994) and appears to be involved in regulation of placental trophoblast differentiation/invasion of the uterus during pregnancy (Caniggia et al., 1997). In PE, trophoblast cells fail to adequately invade maternal spiral arteries, converting them from small muscular vessels to large low resistant vessels. Without this conversion, arterial lumen diameter/distensibility is limited, leading to reduced placental and fetal perfusion (Brosens, Robertson, & Dixon, 1967; Brosens, Roberston, & Dixon, 1972; Gerretsen, Huisjes, & Elema, 1981; Khong, De Wolf, Robertson, & Brosens, 1981; Zhou, Damsky, Chiu, Roberts, & Fisher, 1993; Zhou, Damsky, & Fisher, 1997). The mechanisms responsible for reduced placental and fetal perfusion secondary to abnormal placentation are presently unknown; however, ENG's role in placental implantation lends support to its potential involvement in PE. Moreover, research examining placental mRNA levels in women with/without PE has demonstrated that first/third trimester placental samples of women who went on to develop PE had significantly elevated levels of *ENG* expression (Farina et al., 2008; Nishizawa et al., 2007; Sitras et al., 2009; Toft et al., 2008).

In addition to abnormal placentation, vascular endothelial function is altered in PE (ACOG, 2002). ENG, which is expressed on vascular endothelial cells (Gougos & Letarte, 1990), has been shown to be involved in the maintenance of vascular tone via the regulation of nitric oxide-dependent vasodilation (Jerkic et al., 2004; Toporsian et al., 2005). Normally, endothelial nitric oxide synthase (eNOS) receives activation signals when the TGFβ1 ligand binds to its receptor complex (Venkatesha et al., 2006). However, a placentally derived, soluble

form of ENG (sENG) found to be significantly elevated in the blood of women with PE (Kim et al., 2009; Levine et al., 2006; Lim et al., 2009; Masuyama et al., 2007; Rana et al., 2007; Robinson et al., 2007; Romero et al., 2008; Salahuddin et al., 2007; Stepan et al., 2008) may impair TGF-β1 ligand binding (Venkatesha et al.). As a result, it is believed that the sequestering of TGF-β1 by elevated levels of sENG affects downstream signaling of other genes and may contribute to the observed clinical sequelae (Venkatesha et al.).

Despite these findings, research of the *ENG* pathway at the molecular level in PE is lacking. A study by Srinivas, Morrison, Andrela, and Elovitz (2010), which explored the association of allelic variation in the closely related angiogenic pathway with PE, failed to find a significant association between *ENG* and PE. Because evaluation of *ENG* in the context of the *ENG* pathway has not been completed, such an investigation has the potential to explain variability in susceptibility to PE, improve understanding of PE, and assist in the development of interventions.

1.2.3 *ENG* pathway candidate genes

The following table lists the *ENG* pathway genes selected for evaluation in this study along with the rationale for their inclusion.

Table 1. Endoglin Pathway Candidate Genes

Gene	Rationale for Inclusion
ENG	Co-receptor of TGFβ1; involved in regulation of placental trophoblast differentiation/invasion and vascular tone (Caniggia et al., 1997; Cheifetz et al., 1992; Jerkic et al., 2004; Toporsian et al., 2005)
TGFβ1	Ligand bound by ENG; involved in regulation of proliferation, differentiation, and migration of many cell types, including trophoblasts. In endothelial cells, downstream signaling leads to *eNOS* expression (Jerkic et al., 2004; Jones, Stoikos, Findlay, & Salamonsen, 2006; National Center for Biotechnology Information [NCBI], 2010; OMIM, 2010; Toporsian et al., 2005; Santibanez et al., 2007); *TGFβ1* mRNA levels (11 week chorionic villous) significantly higher in women who later developed preeclampsia compared to healthy controls (Farina et al., 2008)
ALK1 (ACVRL1) *ALK5 (TGFβR1)*	Type 1 receptor of TGFβ1;forms a heterodimeric complex with Type 2 receptor (TGFβR2); responsible for transmission of TGFβ1 signals; interacts with ENG (Guerrero-Esteo et al., 2002; OMIM, 2010; ten Dijke et al., 2008)
TGFβR2	Type 2 receptor of TGFβ1; forms a heterodimeric complex with Type 1 receptors; responsible for transmission of TGF-β1 signals; interacts with ENG (Guerrero-Esteo et al., 2002; OMIM, 2010; ten Dijke et al., 2008)
SMAD1, 2, 3, 4, 5, 6, 7, 8	Proteins involved in transmission of TGFβ1 signals from cell-surface receptors to the nucleus (OMIM, 2010; ten Dijke et al., 2008)

1.2.4 Conceptual framework

The following figure diagrams the conceptual framework that guided this study, which examined the association between the genetic composition of the *ENG* pathway and the development of preeclampsia while accounting for potential covariates (e.g., age, race).

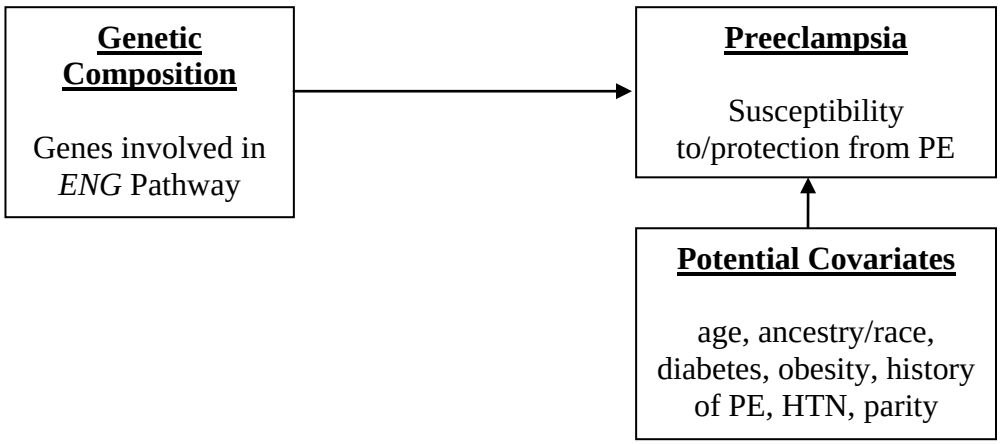

Figure 1. Conceptual Framework

1.2.5 Potential covariates

The development of multi-faceted disorders, including PE, can be influenced by genetic and environmental factors. Although the proposed pathway specific, candidate gene association study will focus on the association between the genetic composition of the *ENG* pathway and susceptibility to/protection from PE, other risks factors may contribute to PE development. In attempt to control for the potential effects of several covariates, healthy controls will be frequency matched (1:1) to cases on maternal age, ancestry/race, and parity. The following table provides rationale for matching on maternal age, ancestry/race, and parity.

Table 2. Covariates

Risk Factor	Supporting Literature
Age	PE is more common at extremes of reproductive age (ACOG et al., 2010; Funai, Evans, & Lockwood, 2008; London, Ladewig, Ball, & Bindler, 2003) Primiparous women ≥ 40 are 1.68 times as likely to develop PE compared to primiparous women < 40 (note: cohort study failed to control or address differences at baseline; e.g., preexisting HTN or diabetes) (Duckitt & Harrington, 2005) Multiparous women ≥ 40 are 1.96 times as likely to develop PE compared to multiparous women < 40 (note: cohort study failed to control or address differences at baseline) (Duckitt & Harrington)
Ancestry	PE more likely to occur in African American women (ACOG et al., 2010; London, Ladewig, Ball, & Bindler, 2003)
Parity	Nearly 2/3rds of PE pregnancies occur in nulliparas (Funai, Evans, & Lockwood, 2008) Nulliparous women are 2.91 times as likely to develop PE compared to multiparous women (Duckitt & Harrington, 2005)

Note. All citations labeled (Duckitt & Harrington, 2005) are presented as unadjusted relative risks

1.2.6 Summary

Despite decades of research, preeclampsia continues to significantly impact the lives of mothers and their fetuses/neonates worldwide. In order to improve short/long term health outcomes and reduce healthcare costs associated with preeclampsia, a more comprehensive understanding of preeclampsia's etiology is needed. Because previous research has demonstrated endoglin's biological plausibility in the development of preeclampsia, further investigation of endoglin is

warranted. Given that preeclampsia most likely represents a polygenic disorder, investigation of endoglin and other genes in its pathway may generate a more detailed representation of the endoglin pathway's potential role in preeclampsia development.

1.2.7 Significance and innovation

This project targets the *ENG* pathway at the molecular level to investigate the impact of genetic variation in the *ENG* pathway of PE. This proposed project is innovative in the following ways:

1. Study of the *ENG* pathway in PE at the molecular level is novel.
2. Results from the project will add to the knowledge base of PE pathogenesis, potentially resulting in clinically relevant biomarkers of susceptibility to PE and early identification of at risk individuals.
3. The project supports the National Institute of Nursing Research's (NINR) research emphasis of promoting health and preventing disease through identification of susceptibility genes for at-risk individuals.

1.3 PRELIMINARY STUDIES

The following table lists several milestones that have been achieved since entrance into the BSN to PhD program in September of 2007. All milestones listed support the feasibility and scientific merit of the proposed dissertation research project titled "Genomics of Endoglin Pathway in Preeclampsia."

Table 3. Milestones

Milestone	Date
PEPP committee approval of submitted research proposal: granted access to de-identified biological samples and data from the PEPP research study	January 22, 2009
Ruth F. Kirschstein National Research Service Award (1F31NR011379) for proposed dissertation research (Genomics of Endoglin Pathway in Preeclampsia)	July 7, 2009
Receipt of PEPP de-identified biological samples (maternal DNA aliquots, fetal cord serum aliquots, and placental samples are stored in the School of Nursing Molecular Genetics Laboratory)	October 2, 2009
University of Pittsburgh IRB Approval (expedited review)	December 2, 2009
Sigma Theta Tau International Honor Society of Nursing, Eta Chapter Research Award (Genomics of Endoglin Pathway in Preeclampsia)	May 28, 2010
Renewal of Ruth F. Kirschstein National Research Service Award (1F31NR011379): year #2	July 19, 2010
IRB Renewal Approval	September 16, 2010
International Society of Nurses in Genetics Research Grant (Genomics of Endoglin Pathway in Preeclampsia)	October 13, 2010

1.3.1 DNA extraction

In addition to these milestones, DNA extraction from the 300 ul fetal serum samples provided by PEPP has been initiated. Using the QIAGEN QiAMP DNA Mini Kit, a total of N = 466 fetal samples have been processed as of November 2010. DNA yield of the extracted samples has

been assessed via Taqman allelic discrimination. Of the extracted samples tested for DNA quality, 92.6% of the samples have passed quality checks.

1.3.2 Preliminary matching

Preliminary frequency matching (1:1) of healthy controls to cases (preeclampsia, severe preeclampsia, or HELLP syndrome) on maternal age, maternal race, and parity further supports the feasibility of the proposed study design.

1.3.2.1 Matching procedures

The following procedures were conducted as part of the 1:1 frequency matching. Controls were matched to cases with the same self-reported race (Black/White) and parity. Although the majority of controls were matched to cases with the same maternal age, seven controls were matched to cases within two years of age due to the lack of healthy women with the same maternal age. One case was excluded from analysis due to the lack of a healthy control subject with a comparable maternal age (+/- 2 years), resulting in the matching of 219 healthy controls to 219 cases. The majority of healthy control subjects (n = 201) were randomly selected and matched to cases (n = 201) for a particular maternal age, race, and parity combination while the remaining cases (n = 18) were directly matched with appropriate control subjects (n = 18).

1.3.2.2 Preliminary matching results

Table 4. Preliminary Matching Results

Characteristic	Cases (*N* = 219)	Controls (*N* = 219)
Maternal Age (*M (SD)*, years)	27.3 (6.2)	27.4 (6.2)
Nulliparous (%)	81.3	81.3
Race		
White (%)	85	85
Black (%)	15	15

1.4 RESEARCH DESIGN AND METHODS

This study will investigate how variation in the *ENG* pathway may relate to genetic susceptibility to/protection from PE using a nested, case/control, candidate gene association design. De-identified genetic samples have been obtained from the Prenatal Exposures and Preeclampsia Prevention cohort Study (PEPP) and tagging single nucleotide polymorphisms (tSNPs) of genes in the *ENG* pathway will be genotyped and analyzed with respect to the specific aims. All aspects of PEPP have been approved by the University of Pittsburgh and Magee-Womens Hospital IRB, including the use of samples/data for genetically-based research. Permission to access these samples was granted by PEPP's Advisory Committee and IRB approval was obtained for this dissertation study. The timeline for this study, which was submitted to the National Institute of Nursing Research as part of the candidate's F31 2010 grant renewal paperwork (1F31NR011379), is included below.

Table 5. Study Timeline

Adjusted Timetable For Project	Year 1	Year 2	Year 3
Coursework			
DNA extraction from fetal cord blood samples			
Conduct bioinformatics for SNP selection and design custom genotyping panes			
DNA dilutions and Whole Genome Amplification			
Collection of genotyping data			
Analysis of data			
Preparation of manuscripts			
Defend dissertation			

1.4.1 Setting and sample

The Prenatal Exposures and Preeclampsia Prevention cohort study (PEPP), which is conducted at Magee-Womens Hospital of UPMC (Pittsburgh, Pa), examines factors predisposing women to PE via two recruitment approaches. The proposed study will utilize samples/data collected from the first two cohorts. In PEPP's first two cohorts, women 14-44 years of age were recruited/enrolled during early pregnancy (≤ 20 weeks' gestation) and followed through delivery/postpartum period at Magee-Womens Hospital or they were recruited/enrolled at the labor/delivery unit of Magee-Womens Hospital due to a suspected diagnosis of preeclampsia. Women with a history of chronic renal disease, hypertension, diabetes, or other disorders that increased the risk of preeclampsia were excluded from longitudinal enrollment. Cross-sectionally enrolled women found to have the aforementioned conditions were excluded from any analyses involving women with preeclampsia and healthy controls.

Demographic, clinical, and laboratory data were collected by trained interviewers. Genomic DNA was extracted from peripherally collected venous blood samples via protein

precipitation from white blood cell pellets. All aspects of PEPP, including the use of samples/data for genetically-based research, were approved by the University of Pittsburgh and Magee Womens Hospital Institutional Review Board (IRB). Separate University of Pittsburgh IRB approval was also granted for this nested study. PEPP subjects not consenting to future genetic evaluation and subjects without a stored genetic sample were excluded from this study.

1.4.1.1 Phenotype definitions

Designation of pregnancy outcome is based on review/discussion of clinical data by a clinical expert panel. The case group includes subjects diagnosed with either preeclampsia (PE), severe PE, or HELLP syndrome while the control group includes healthy subjects without past medical histories.

1. **Cases:**

   PE was based on blood pressure (BP), urinary protein, and serum uric acid criteria. Hypertension (HTN) = BP ≥ 140 mmHg systolic and/or 90 mmHg diastolic AND an increase of BP > 30 mmHg systolic and/or 15 mmHg diastolic after 20 weeks' gestation, which were based on the average of the last five BPs taken in the hospital prior to therapeutic intervention (medication; anesthesia) compared to the average BP prior to 20 weeks' gestation. BP abnormalities were to resolve by 12 weeks postpartum. Proteinuria = ≥ 300 mg/ 24hours, ≥ 0.3 protein/creatinine ratio, ≥ 2+ on a random urine specimen, or ≥ 1+ on a catheterized urine specimen. Hyperuricemia = serum uric acid concentration > 1 standard deviation from normal for gestational age.

Severe PE = PE + ≥ 1 of the following conditions: (1) systolic BP ≥ 160 mmHg; (2) diastolic BP ≥ 110 mmHg; (3) proteinuria ≥ 5 grams/ 24 hours; (4) elevated liver enzymes; (5) platelet count ≤ 100,000

HELLP syndrome = PE + presence of hemolysis, elevated liver enzymes, and low platelets

2. **Controls:**

Women who were clinically evaluated and did not meet the above criteria for PE

1.4.1.2 Sample breakdown and matching

The proposed study sample consists of 1473 maternal subjects (*n* = 225 cases; *n* = 1248 controls) with banked samples, demographic, clinical, and laboratory data. There are 1027 maternal subjects classified as White, 405 classified as Black, 8 classified as Hispanic, 22 classified as Asian/Pacific, 2 classified as Native American, and 9 classified as "Other". A total of 1493 fetal/neonatal samples (222 from case pregnancies; 1271 from control pregnancies), along with demographic and clinical data are also available (Note: 23 women were enrolled in PEPP for two different pregnancies. A fetal sample from the second enrollment was also provided). Controls will be frequency matched (at least 1:1) to cases on ancestry, maternal age, and parity. Other potential confounders and covariates detected in the preliminary analyses will be controlled for in the primary analyses.

1.4.2 Polymorphism selection for assessment of candidate genes

Single nucleotide polymorphisms (SNPs) having the potential to be functional based on the scientific literature will be included in the assessment of the candidate genes when appropriate. Because these SNPs will not fully evaluate a gene, tSNPS for each gene will be selected via the HapMap database (www.hapmap.org) in order to fully evaluate the genetic variability of the candidate genes with the least number of SNPs. Promoter regions of the candidate genes will also be evaluated within the context of the tSNPs. Tagging SNP selection criteria includes:

1. Minor allele frequency $\geq$ 20% for each tSNP
2. R^2 cutoff = .80
3. Caucasian (CEU) and African (YRI) ancestry

Due to the dynamic nature of the HapMap database, tSNP selection will be re-evaluated immediately prior to the initiation of genotyping efforts.

1.4.3 Genotype data collection

The i-PLEX® Gold SNP Assay (Sequenom® Inc, San Diego, CA) will be used for genotype data collection by the University of Pittsburgh Genomics and Proteomics Core Laboratories (http://www.genetics.pitt.edu/). Several steps are sequentially followed to determine SNP genotypes, whose allelic compositions are differentiated based on differences in molecular mass via matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Sequenom). This is an appropriate methodology given the number of SNPs to be evaluated

and has the advantage of multiplexing therefore reducing assay to assay variability as well as reducing costs. Steps one through four are listed below:

1. Primer Design: Three primers are designed for each SNP of interest using MassARRAY® Assay Design 3.1. The two amplification primers flank the polymorphic site to provide for sample amplification, while the single MassExtend primer lies immediately adjacent to allow for allelic discrimination via single base extension. Assay Design software determines how primer sets can be pooled to optimize multiplex reactions. Mass modifications are incorporated in the design of the MassExtend primers to maximize the mass differential between primers of different loci within a given multiplex pool.

2. Sample Amplification: Target loci are amplified within the samples by multiplex PCR in 1X PCR buffer (Qiagen) containing 3.5 mM $MgCl_2$, 25 mMdNTPs, 500 µM each forward and reverse amplification primer within the multiplex pool and 2.5 U HotStarTaq (Qiagen). PCR conditions are: 95^{o}C for 15 minutes for taq activation followed by 45 cycles of 94^{o}C for 20 seconds, 56^{o}C for 20 seconds and 72^{o}C for 1 minute. A single extension for 1 minute at 72^{o}C completes the PCR reaction. dNTPs and primers are removed by incubation with 0.5 U shrimp alkaline phosphotase (SAP) at 37 ^{o}C for 40 minutes. SAP is inactivated by incubation at 87 ^{o}C for 5 minutes.

3. MassExtend: Excess MassExtend primers corresponding to the loci represented by the amplification primers used are pooled. Higher mass primers are added at a higher concentration to adjust for signal drop off during spectra acquisition. Single base extension is carried out in 0.2X iPLEX buffer plus, 1X termination mix (containing mass

modified termination nucleotides), 1X iPLEX enzyme and primers at 0.84 μM, 1.04 μM and 1.25 μM as appropriate to the relative mass of the primer. A double cycle amplification program performs 40 cycles of denaturation at 94 ^{o}C for 5 seconds followed by 5 cycles of 52^{o}C for 5 seconds, 80 ^{o}C for 5 seconds, back to 94 ^{o}C for a total of 200 cycles. A final extension at 72 ^{o}C for 3 minutes completes the amplification. Clean resin and water is added to the MassExtend reaction products. Samples are incubated in clean resin at room temperature with mixing for 5 minutes and centrifuged at 3200 x g for 5 minutes.

4. Nanodispense, Spectra Acquisition and Analysis: Samples are dispensed to a SpectraChip using the MassARRAY® Nanodispenser according to manufacturer's instructions. Spectra chips are loaded into the MassARRAY® analyzer and spectra acquired for each sample. MassARRAY® Typer software uses the known mass of the MassExtend primers to identify each locus, and the increase caused by each distinct nucleotide to identify the alleles present in the sample.

1.4.4 Reliability of genotype data and haplotype assignment

Reliability of genotype data will be evaluated in several steps, with attention focused on validation of data for consistency and integrity. Steps include checks comparing expected homozygosity to observed homozygosity at each SNP, checks of allele frequencies, consideration of genotype call rates, and checks for Hardy-Weinberg Equilibrium consistency. Haplotypes will be developed and analyzed in addition to the independent evaluation of each SNP. The HAPLO.CC Function of the HAPLO.STATS Package (Version 1.2.2) of the R

Genetics Package (Schaid, Rowland, Tines, Jacobson, & Poland, 2002) will be used to conduct 2- and 3- SNP moving window haplotype analyses for each candidate gene. Haploview (Version 3.32) (Barrett, Fry, Maller, & Daly, 2005) will be used to estimate linkage disequilibrium across each candidate gene for both D' and R^2.

1.4.5 Analysis

1.4.5.1 Sample size justification

Quanto version 1.2.4 was used to conduct a power analysis for this genetic association study. Information for the following program parameters were entered: (1) SNP minor allele frequency: 0.2 - 0.5; (2) no environmental influence; (3) additive gene effect due to preeclampsia's multi-factorial nature; (4) 1:1 case/control matching based on study design; (5) population risk of 5% (Roberts & Cooper, 2001); (6) heritability factor range 0.31 - 0.54 (Nilsson, Salonen Ros, Cnattinguis, & Lichtenstein, 2004; Salonen Ros, Lichtenstein, Lipworth, & Cnattingius, 2000); (7) α =.05; and (8) two-sided test. A sample of 225 cases resulted in statistical power ranging from 0.9496-0.9999. Although some assumptions may be incorrect and no correction was made for multiple testing, these calculations demonstrate that 225 cases allows for analysis of each gene separately for aim 1. Aim 2 is exploratory in nature; however, with the increased power of the TDT approach and the availability of 225 affected maternal/fetal dyads, it is believed that data generated will indicate significant associations when they truly exist.

1.4.5.2 Preliminary analysis

Prior to analysis of data generated in relation to the specific aims, descriptive statistics of the grouped data (cases and controls) will be computed to describe the data and sample distributions. Frequency counts, percentages, modes (for nominally and ordinally scaled variables), and medians (for ordinally scaled variables) will be generated to describe categorical variables. The range will be used to describe the variability of nominal variables, while the interquartile range and semi-quartile range will be used to describe variability of ordinal data. Means, medians, modes, standard deviations, frequencies, percentages, skewness, and kurtosis will be computed for all continuous-type ratio variables.

Grouped data screening procedures will also be employed prior to analysis of the specific aims in order to appraise data accuracy, assess missing data, detect outliers, evaluate underlying assumptions, transform data, and check the reliability of genotype data. Data accuracy will be appraised via range checking. If abnormalities are identified, questionable observations will be reviewed to determine if the entered values are valid or are due to errors in data entry or chart abstraction. If values are found to be a result of incorrect data entry or chart abstraction, the data manager of PEPP will be contacted to obtain the correct values.

The amount of missing data, patterns of missing data, and the missing data mechanisms will be investigated using the Missing Values Analysis (MVA) function in SPSS. Depending on the amount, pattern, and mechanism of the missing data, several options for handling the missing data will be considered. Such options include omission of the variable from analysis, complete-case analysis, available-case analysis, and imputation. If possible, regression, expectation-maximization, or multiple imputation will be utilized to estimate the missing values. For the genetic data, SNPs with a genotype call rate of < 86% will be omitted from analysis (Y. Conley,

personal communication, March 31, 2010). For SNPs included in the analysis, available-case analysis will be utilized given that missing genotype values are typically missing completely at random. Furthermore, no attempt will be made to impute genotype values, for it is not clear what subject predictors could be used to impute SNP genotypes.

The grouped data will also be screened for univariate and multivariate outliers. For univariate, categorical outliers, variables with uneven splits among the categories (e.g., 90—10 for dichotomous variables) will be detected via frequency analysis. Histograms, boxplots, normality probability plots, and detrended expected normal probability plots will be generated to assess univariately for outliers for continuous variables. Z scores will also be computed to identify potential univariate outliers and bivariate scatterplots will be generated to assess for multivariate outliers. Data transformations, such as score alterations, will also be considered to decrease the influence of outliers. Any data transformations will be noted in any reports or papers.

Parametric or non-parametric tests (e.g., two-sample t-tests, chi-square test of independence, Mann-Whitney U-test) will also be conducted, as appropriate, to compare cases and controls on additional continuous and categorical variables in an attempt to identify extraneous covariates or possible confounders. Such variables include income, education, delivery method, body mass index (BMI), infant weight, and blood pressure. Prior to these parametric data analyses, assessment of underlying assumptions will first be performed on the groups for continuous variables (income, education, BMI, infant weight, blood pressure). Measures of skewness and kurtosis will be generated along with frequency histograms with normal distribution overlays and the Kolmogorov-Smirnov or Shapiro-Wilk tests to assess the assumption of normality. The assumption of linearity will be evaluated via bivariate scatterplots

and Levene's test will be used to assess for homoscedasticity. Finally, to fulfill the assumption of independent observations, only subject data from a single enrollment in PEPP will be analyzed. However, if the data are found to be non-normally distributed, non-linear, or heteroscedastic, data transformations will be attempted to induce normality, enhance linearity, and stabilize variances. If the statistical assumptions remain severely violated, non-parametric tests will be used.

The data will also be screened to assess assumptions related to binary logistic regression, which represents the statistical approach to be used to address specific aim #1. Although some assumptions will be evaluated as part of the logistic regression analysis procedure, checks for multicollinearity and sparseness of cells will be conducted prior to analysis. To assess for multicollinearity in the predictor variables, tolerance and variance inflation factor conditioning indices will be computed. If multicollinearity is found, an attempt will be made to drop the collinear variables or create a new variable that is a function of the collinear variables. In addition, the potential issue related to sparseness of cells in the categorical variables will be examined via frequency counts. If sufficient data within different levels of the categorical predictors are found to be lacking, variable categories will be collapsed to limit the sparseness of cells. If cells are unable to be collapsed, such predictors will be omitted from the main analyses, limiting such predictors to descriptive analysis.

Reliability of genotype data will be evaluated in several steps. Particular attention will be focused on validation of the data for consistency and integrity. These steps include: checks comparing expected homozygosity to observed homozygosity at each SNP, checks of allele frequencies, consideration of genotype call rates, and checks for Hardy-Weingberg Equilibrium consistency (check for genotyping error).

1.4.5.3 Aim 1 analysis

Investigate variation in maternal genes involved in the endoglin pathway for impact on the development of PE

The relationship between the presence or absence of preeclampsia and the presence of each allele and/or genotype and/or haplotype will be examined via contingency table analysis with chi-square tests of independence and binary logistic regression analysis. Preeclampsia, a binary variable (present or absent), is the outcome of interest. The independent variables will be noted as the presence of one allele or the other of the genotype (allele 1 or allele 2) and the presence of one genotype or the other (homozygous for allele one, homozygous for allele 2, or heterozygous).

Initially, chi-square tests of independence will be used to test whether there is a relationship between the occurrence of preeclampsia and the presence of each SNP alleles and/or genotype and/or haplotype. Furthermore, because the variables of interest are categorical, binary logistic regression models will be fitted to yield unadjusted odds ratios and their 99% confidence intervals for each candidate gene SNP(s) separately. In order to reduce inflation of type 1 error, the more conservative 99% CI will be computed. To control for potential covariate and confounding effects, the models will be expanded via hierarchical, multivariate logistic regression to yield adjusted odds ratios (99% confidence intervals) and the contribution of genetic susceptibility/protection after controlling for covariates. Identified covariates will be entered into the first block and the candidate gene SNP will be entered into the second block.

After conducting the logistic regression procedures, model fit and satisfaction of underlying assumptions will be assessed. Goodness-of-fit (GOF) will be examined via the Hosmer-Lemeshow GOF test. If any of the identified covariates entered into the model are

continuous in nature, the assumption of linearity in the logit will be assessed using Box-Tidwell approach and subsequent graphical methods if necessary. For the Box-Tidwell approach, interaction terms (cross-product of an independent variable times its natural logarithm) will be created and added to the logistic model. If the interaction terms are found to be significant, non-linearity will be further assessed with graphical methods. The graphical assessment will involve the following steps:

1. Creation of a grouped version of the continuous variable based on quartiles
2. Dummy coding of the grouped version of the continuous variable
3. Entering all dummy variables into the regression model simultaneously
4. Generation of beta coefficients (e.g., log odds)
5. Plotting of the beta coefficients against the midpoint for the group to assess for linearity. Analysis of fitted models will also include residual analysis (identification of outliers in solution, influential observations). If an acceptable model is found, the regression coefficients will then be evaluated for statistical significance via the Wald test and the likelihood ratio chi-square test.

1.4.5.4 Aim 2 analysis

Explore variation in maternal/fetal dyad genes involved in the endoglin pathway for impact on development of preeclampsia.

Because specific aim #2 involves related dyads (mother/infant), transmission disequilibrium testing (TDT) will be used to analyze the genetic data related to specific aim #2. TDT is based on the assumption that there is over-transmission of an allele from parents to affected offspring and under-transmission of that same allele from parents to unaffected

offspring when that allele is implicated in susceptibility (Sun, Flanders, Yang, & Khoury, 1999). For the proposed study, offspring born to women who experience preeclampsia during their pregnancy are considered to be affected since preeclampsia only develops during pregnancy.

Traditionally, TDT involves the analysis of a family trio, which includes both parents and the offspring. In the proposed study, genetic information is only available for one parent and her offspring. However, modifications have been introduced so that TDT can be conducted with only one parent and one offspring (1-TDT). As a result, the genomic contribution of the endoglin pathway amongst the mother/child dyads in the proposed study can be explored using 1-TDT.

Additionally, there is strength in combining the unrelated case-control data from aim 1 with the maternal fetal dyads from aim 2 and we plan to use the CCREL program to conduct these analyses. CCREL is a program for case-control genetic analysis that takes relatedness between individuals into account, allowing one to analyze both related and unrelated individuals at the same time. It will perform single-marker and haplotypic tests for biallelic markers, which is what this study will utilize, and it will allow for the compilation of effect sizes for use in future studies.

1.5 POTENTIAL LIMITATIONS OF PROPOSED PROCEDURES AND ALTERNATIVE APPROACHES TO ACHIEVE THESE AIMS

Several limitations are associated with the proposed study procedures. Because the proposed research study is retrospective, inadequate matching of controls to cases on the variables of

interest along with inadequate minority representation due to an inadequate sample size represents a potential limitation. Such a limitation could result in the findings of the investigation being underpowered, but a strength of the PEPP study is that minorities (Black subjects) are well represented. However, given that population substructure can lead to issues when conducting genetic association studies it may be necessary to analyze populations from different ancestries separately for SNPs where allele frequency differs significantly. If this occurs, it could impact power for that particular SNP, but the matching of cases to controls may help with this issue and would provide pilot data that may aid in understanding of health disparities related to preeclampsia.

Another potential limitation that may be encountered would be inappropriate sample size if too many covariates need to be considered. An alternative approach to address sample size and power issues would be to study additional subjects that may become available from the PEPP 3 cohort, which is currently recruiting and collecting data on subjects. Thus, even though the proposed research may be exploratory, the results would have the potential to support follow-up studies in which the investigator could attempt to rectify the potential sampling limitations that exist when utilizing existing samples.

SNP incompatibility as a result of multiplexing presents another potential limitation. Although the investigator will attempt to develop primer sets in which the number of SNPs are maximized per multiplex assay, there may be some SNPs whose primers will not hybridize and allow for sample amplification in the presence of other SNP primers. An alternative approach would be to genotype these SNPs separately using other methods such as TaqMan®.

The utilization of a nested design may also present a potential limitation. Because the investigator proposes to utilize samples and data from an existing parent study, she cannot

control the types of data that were collected, including information on potential covariates. In the future, an alternative approach would be to design and implement one's own parent study. This approach would allow her to define inclusion/exclusion criteria and choose the variables for which data will be collected.

Finally, the investigator may find that none of the selected genes are significantly associated with PE. Although not optimal, these results would provide the scientific community with valuable information. Such information could potentially contribute to a better understanding of PE pathophysiology.

1.6 HAZARDOUS MATERIALS AND PROCEDURES

While working in the laboratory, I will come into contact with numerous substances, including blood, blood products, and certain chemicals. In order to safely conduct protocols utilizing such substances, I will apply personal protective equipment, including gloves, goggles, a laboratory coat, and a mask as appropriate. Additionally, I have completed the required Bloodborne Pathogen Training and the Chemical Hygiene Training.

1.7 RESEARCH PARTICIPANT RISK AND PROTECTION

1.7.1 Human subjects

IRB approval has been obtained for the proposed study (PR009110136) (Appendix 1). Specimens received for the study have been previously collected from subjects enrolled in PEPP. Because the proposed study is investigating variation in maternal and fetal genes involved in the *ENG* pathway for impact on development of PE, data from pregnant women and fetal subjects are included. Furthermore, because the placenta is thought to be involved in the development of PE and the placenta is of fetal origin, the inclusion of fetal subjects, specifically fetal DNA obtained from cord blood samples, is inherent to the study of PE. Lastly, young age has been indicated as a potential risk factor for the development of PE, which supports the inclusion of pregnant teenagers (children) in the study sample. In reference to the PEPP study sample, pregnant children were included in the study if they were ≥ 14 years of age. Thus, samples from pregnant women, fetuses, and children comprise the PEPP sample and the proposed study sample. Furthermore, minorities have not been excluded from the PEPP or proposed study.

1.7.2 Potential risks and data/safety monitoring

While breach of confidentiality is a concern of genetic studies, the following precautions will attempt to mitigate this risk. First, all biological samples and clinical, demographic, and laboratory data have been obtained from PEPP in a de-identified manner via an honest broker. Neither I nor the laboratory staff will have access to the data linking the subject's ID number to

personal identifiers. Second, all data generated from the proposed study will be stored in a database of a password protected computer or on removable storage that will be stored in locked file cabinets or be password protected. Third, all data generated from the proposed study will be reported as aggregate data and results will not be revealed to subjects. Fourth, genetic and clinical data will be used solely for research purposes and will be continuously safeguarded by myself and my mentor.

1.7.3 Potential benefits

Although results generated will provide no direct benefit to study subjects, findings may increase our knowledge of the underlying pathophysiologic mechanisms involved in PE development. Such knowledge has the potential to serve as a foundation in the design and implementation of interventions aimed at prevention, detection, and treatment of PE.

1.8 PUBLICATIONS

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2.0 SUMMARY OF STUDY

The purpose of this dissertation research was to examine the relationship between endoglin (*ENG*) pathway genetic variation and the development of preeclampsia. Two articles that were written during the course of this dissertation project are provided in Appendices B and D. Published in the *Journal of Obstetric, Gynecologic, & Neonatal Nursing*, the article titled "A Historical Overview of Preeclampsia-eclampsia" (Appendix B) highlights theories on disease causation and reviews changes in the treatment and classification of preeclampsia from Ancient times through the 21st century. The second article titled "A Systematic Review of Endoglin Gene Expression in Preeclampsia," (Appendix D) which is published in *Biological Research for Nursing*, was written to summarize gene expression studies addressing the role of endoglin in preeclampsia and further highlighted the need for the investigation of endoglin at the molecular level. Together, these two articles provided the rationale for this dissertation project. The results for specific aim 1 are presented in the data based manuscript in the section that follows this summary of the study. A discussion of study aim 2 is provided below.

2.1 PROPOSAL CHANGES

Throughout the course of this project, several changes were made to the approved dissertation proposal. The changes, along with the rationale for these changes, are provided below. A separate section addressing the decision to forgo the analysis of study aim 2 is presented after discussion of the other proposal changes.

2.1.1 Candidate genes evaluated

This study originally proposed to evaluate 13 *ENG* pathway candidate genes (*ENG*, *TGFβ1*, *ALK1*, *TGFβR1*, *TGFβR2*, and *SMADs 1-8*). Based on a HapMap (Data Phase III/Rel#2, Feb09, on NCBI B36 assembly, dbSNP b126) query conducted on March 24, 2011, 117 tSNPs across the 13 candidate genes were needed to fully evaluate their genetic variability. Due to the large number of tSNPs and the financial costs implicated with genotyping such a large number of tSNPs, the decision was made to exclude candidate genes from this study. Because the *SMAD* genes were more extrinsic to the pathway and a total of 70 tSNPs needed to be genotyped to fully evaluate these genes, the *SMAD* genes were excluded from this study. With this exclusion of these genes, the number of tSNPs to be analyzed across *ENG*, *TGFβ1*, *ALK1*, *TGFβR1*, and *TGFβR2* totaled 47 tSNPs, which required two separate iPLEX® assays to accommodate this reduced number of SNPs.

2.1.2 Tagging SNP selection criteria

This study originally proposed to select tSNPs for both Caucasian (CEU) and African (YRI) ancestry. Because the majority of this study's sample was comprised of Caucasian subjects, the decision was made to only select tSNPs for Caucasian ancestry. We do acknowledge that linkage disequilibrium in the candidate genes may be different for Caucasian and African ancestries, thereby decreasing the informativeness of the data generated in our our black subgroup; however, we knew that our analysis in the black subgroup would be exploratory in nature given the small sample size.

2.1.3 iPLEX® assay design and potentially functional SNPs

Since the writing of the proposal, the versions of the software utilized to design iPLEX® assays have been updated. The updated software, which includes the online Human GenoTyping Tools and the MassARRAY® Designer v4.0 software, was used to design two multiplex assays. Because the tSNPs from *ENG*, *TGFβ1*, *ALK1*, *TGFβR1*, and *TGFβR2* were of highest priority, the assays were designed to first include these tSNPs. Two potentially functional SNPs that did not interfere with the assays' abilities to genotype the candidate gene tSNPs were also included.

2.1.4 Genotype data collection with TaqMan® allelic discrimination

Originally all of the genotype data was to be generated using iPLEX®; however, five SNPs did not meet the data quality criteria with the iPLEX® platform either because call rates were < 86%

(3 SNPs) or the SNPs were multi-allelic (3 allele and 4 allele). For these SNPs, pre-developed and commercially available TaqMan® SNP Genotyping assays (Applied Biosystems®) were used to conduct TaqMan® allelic discrimination with the ABI Prism® 7000 Sequence Detection System and SDS software v1.2.3 (Applied Biosystems Inc., Carlsbad, CA).

2.1.5 Haplotyping and linkage disequilibrium estimation

We originally proposed to estimate haplotypes and estimate linkage disequilibrium for all candidate genes using the HAPLO.STATS Package (version 1.2.2) of the R Genetics Package and Haploview (version 3.32) (Barrett, Fry, Maller, & Daly, 2005). Instead, we used PLINK software (version 1.07) (Purcell et al., 2007), which was capable of completing both of these tasks. Because of the small number of black cases and controls (30/30) and the use of only Caucasian ancestry to select tSNPs, we did not conduct haplotype analysis or estimate linkage disequilibrium in blacks. In the white subgroup, we decided to only conduct haplotype analyses and estimates of linkage disequilibrium in *ENG* because of significant associations at the allele and genotype level. Although *TGFβR2* had a significant allele and genotype test for one tSNP, we did not estimate haplotypes or linkage disequilibrium given the large number of tSNP (32) data that would need to be utilized in conducting the haplotyping.

2.1.6 Statistical changes to Aim 1

Several statistical changes were made during the analysis of aim 1. First, binary logistic regression was only utilized to compute odds ratios in tSNPs that had significant genotype tests.

We felt that the computation of odds ratios for those tSNPs with non-significant genotype tests was not needed given the lack of association. Second, we decided not to conduct predictive modeling via hierarchical multivariate logistic regression since our study's purpose was not aimed at prediction. Third, we identified clinical characteristics (e.g., blood pressure, pre-pregnancy BMI) that were significantly different among cases and controls. We then tested the association between those clinical characteristics and genotype assignment for tSNPs found to have significant genotype tests in the white and black subgroups separately.

2.2 PROPOSAL CHANGE: STUDY AIM 2

The purpose of this study's second aim was to explore how variation in endoglin (*ENG*) pathway genes (*ENG*, *TGFβ1*, *TGFβR1*, and *TGFβR2*) of maternal/fetal dyads may be associated with the development of preeclampsia. Tagging single nucleotide polymorphisms (tSNPs) and potentially functional SNPs identified in the literature were selected to evaluate candidate gene variability in mother/fetal dyads with a preeclampsia diagnosis (cases) compared to healthy maternal/fetal dyads (controls). Maternal and fetal biological samples along with demographic, clinical, and laboratory data were provided by the Prenatal Exposures and Preeclampsia Prevention study (PEPP) conducted at Magee-Womens Hospital of UPMC (Pittsburgh, Pa). A total of 215 maternal controls were 1:1 frequency matched to 215 maternal cases on age (+/- 2 years), ancestry (black/white), and parity. For maternal subjects, PEPP provided genomic DNA aliquots (100 μl). The genomic DNA samples had been extracted from peripherally collected blood samples via protein precipitation from white blood cell pellets and passed our quality

assessment checks. For fetal subjects, PEPP provided serum aliquots (300 µl) obtained from umbilical cord blood after delivery. In order to conduct aim 2, we first needed to extract genomic DNA from the fetal serum samples and assess DNA yield, quality, and utility of the extracted samples.

2.2.1 DNA extraction and DNA yield/quality assessment of fetal samples

QIAGEN QIAamp® DNA Mini Kits (QIAGEN, Valencia, CA) were used to extract genomic DNA from 300 µl umbilical cord serum aliquots per manufacturer's instructions. TaqMan® allelic discrimination (Applied Biosystems Inc., Carlsbad, CA) was utilized to assess DNA yield, quality, and utility of extracted samples. We used the following thermal profile: (a) AmpliTaq Gold Enzyme activation at 95°C for 10 minutes, (b) denature at 95°C for 15 seconds, (c) anneal/extend at 58°C for 1:30 minutes, (d) go to step b 50 times, (e) hold at 10°C. For those fetal samples with initial insufficient yield, whole genome amplification was carried out with the REPLI-g® Midi Kit per manufacturer's instructions (QIAGEN, Valencia, CA) to increase available template for investigation. These samples, once amplified, were subjected to the same TaqMan® assessment to ensure utility of the samples.

2.2.2 Genotyping methods for fetal samples

The iPLEX® Gold-SNP Genotyping assay (Sequenom® Inc, San Diego, CA) was utilized to genotype the 49 SNPs. Two multiplex assays consisting of two amplification primers and one

single base extension primer for each SNP locus were designed with the online Human GenoTyping Tools and MassARRAY® Designer v4.0 software (https://www.mysequenom.com). Genotyping was conducted at the University of Pittsburgh Genomics and Proteomics Core Labratories using the MassARRAY® Nanodispenser (Samsung, Irvine, CA), MassARRAY® Compact Analyzer (Bruker, Newark, DE), SpectroACQUIRE v3.3.1.3 software (Sequenom®), and MassARRAY® Typer v4.0 software (Sequenom®) (http://www.genetics.pitt.edu/).

Five SNPs did not meet data quality criteria with the iPLEX® platform. Three SNPs (rs1800468, rs10739778, rs6809777) had genotype call rates of < 86% across both assays (looking at maternal and fetal data together) and two SNPs (rs3087465, rs8179181) were multi-allelic (>2 alleles), causing genotype data validity to be questioned. For these five SNPs, we performed TaqMan® allelic discrimination with the ABI Prism® 7000 Sequence Detection System and SDS software v1.2.3 (Applied Biosystems Inc., Carlsbad, CA), using pre-developed and commercially available TaqMan® SNP Genotyping assays (Applied Biosystems®). We used the following thermal profile: (a) AmpliTaq Gold Enzyme activation at 95°C for 10 minutes, (b) denature at 95°C for 15 seconds, (c) anneal/extend at 58°C for 1:30 minute, (d) go to step b 50 times, (e) hold at 10°C. Genotype data collection for these five SNPs was collected at the University of Pittsburgh School of Nursing Molecular Genetics Laboratory.

2.2.3 Assessment of genotype reliability in fetal samples

The following steps were taken to assess genotype reliability for the white subgroup fetal samples. The starting sample was comprised of 362 fetal samples due to the removal of a

maternal/fetal dyad from the case group (poor maternal sample call rate) and a randomly selected maternal/fetal dyad from the control group.

1. Global assessment of 49 SNP call rates across the two iPLEX® assays (maternal and fetal data collectively analyzed) and removal of 5 SNPs that were multi-allelic or had global call rates < 80% on both assays
2. Assessment of fetal sample call rates across the two iPLEX® assays and removal of 32 fetal samples with global call rates < 80% on both assays.
3. HWE checks were performed for 41 of the 44 remaining SNPs in 330 fetal subjects. Of the three SNPs not evaluated, one was monomorphic and two had call rates of < 12% when just looking at the fetal data.
4. Fetal subjects born prematurely in the control group were removed from the sample and SNP call rates were re-evaluated in the fetal sample. SNPs with call rates < 80% in just fetal samples were removed.
5. Call rates of the remaining 324 fetal samples were re-evaluated and those samples with call rates < 86% were removed.
6. HWE checks were started for the 38 remaining SNPs in the 284 remaining fetal samples, but were stopped to evaluate the fetal sample for potential segregation issues (e.g., maternal genotype = CC and fetal genotype = TT).
7. Fetal samples with segregation issues on at least 1 of the 38 SNPs were noted.
8. Three of the five SNPs not able to be genotyped with iPLEX® were successfully genotyped with TaqMan®. Sample call rates were re-evaluated after inclusion of these three SNPs and those samples with call rates < 86% across the 41 SNPs were removed. These three SNPs were also analyzed for segregation issues.

9. Umbilical cord serum aliquots (500 μL) for fetal samples with segregation issues on ≥ 2 SNPs were obtained and genomic DNA was extracted with QIAGEN QIAamp® DNA Midi Kits (QIAGEN, Valencia, CA).
10. TaqMan® allelic discrimination was used to compare maternal genotype, fetal genotype from the initial sample, and fetal genotype from the new sample.
11. Samples with segregation issues on ≥ 2 SNPs were removed and HWE was checked for 40 of the 41 SNPs in the remaining 255 fetal subjects. (Note: one SNP had expected frequency counts < 5 with the chi square test of independence and therefore needed to be checked with an exact test).
12. Samples with segregation issues on just 1 SNP were removed and HWE was re-checked for 40 of the 41 SNPS in the remaining 219 fetal subjects.

A similar assessment was made in the black fetal subgroup. First, 49 SNP call rates were globally assessed across the two iPLEX® assays (maternal and fetal data collectively analyzed) and 5 SNPs that were either multi-allelic or had global call rates < 80% on both assays were removed. Second, the assessment of fetal sample call rates across the two iPLEX® assays resulted in the removal of 1 fetal sample with a global call rate < 80% on both assays. Third, HWE was evaluated in 19 of the 44 SNPs in 59 fetal subjects (18 SNPs could not be assessed with a chi square test of independence, 6 SNPs had call rates of < 86%, and 1 SNP was basically monomorphic in the black fetal samples). Given these results and what we were observing in the white fetal subgroup, the decision was made to evaluate the fetal samples for segregation issues, as noted in steps 9 and 10. Addition of 3 tSNPs genotyped with TaqMan®, resulted in the evaluation of segregation issues for 40 SNPs across 56 dyads (3 fetal samples removed for

sample call rates < 86%). Based on those results, no further analysis of the black fetal sample was conducted.

2.2.4 Results in white fetal subgroup

The first round of HWE checks (step 3) found that 32 of the 41 SNPs (78.0%) were out of HWE in 330 white fetal subjects. A closer evaluation of fetal genotype data compared to maternal genotype data revealed that there were segregation issues for 63 maternal/fetal dyads, with 26 dyads having segregation issues on ≥ 2 SNPs. After removal of fetal subjects with segregation issues on ≥ 2 SNPs and the removal of subjects with call rates < 86%, re-evaluation of HWE (step 11) showed that 17 out of 40 SNPs (42.5%) were still out of HWE in the reduced sample of 255 fetal subjects. After the removal of subjects with a noted segregation issue on just one SNP, a final round of HWE checks was performed. Despite the removal of all fetal subjects with poor call rates and segregation issues on at least one SNP, 13 of 40 SNPs (32.5%) were out of HWE in the reduced sample of 219 fetal subjects.

In addition to the multiple rounds of sample reduction and HWE checks, fetal samples with segregation issues on ≥ 2 SNPs were further analyzed by comparison of the maternal sample genotype, the original fetal sample genotype, and the new fetal sample genotype. Of the 25 original fetal sample genotypes that we compared to the 25 new fetal sample genotypes with TaqMan® allelic discrimination, 4 (16%) of the original fetal sample genotypes did not match the genotypes of their respective new fetal samples; noting that this was only conducted for one SNP as proof of concept.

2.2.5 Results in black fetal subgroup

In the black fetal sample, 6 of 19 SNPs (31.6%) that could be checked for HWE using a chi square test were out of HWE in 59 subjects. A closer evaluation of fetal genotype data compared to maternal genotype data showed that there were segregation issues for 15 maternal/fetal dyads, with 8 dyads having segregation issues on ≥ 2 SNPs. Of the 7 original fetal sample genotypes that we compared to the 7 new fetal sample genotypes with TaqMan® allelic discrimination, 1 (14.3%) of the original fetal sample genotypes did not match the genotypes of their respective new fetal samples; noting that this was only conducted for one SNP as proof of concept.

2.2.6 Discussion

At the beginning of this study, we were not certain if DNA obtained from serum aliquots would be of high enough yield/quality to move forward with aim 2. We have found that the use of genomic DNA extracted from small-volume aliquots of umbilical cord serum does not appear to represent a high quality template that can be used for genotype data collection with the iPLEX® template. Even after removal of white fetal subjects with poor call rates and segregation issues, 32.5% of the SNPs to be analyzed were out of HWE in the white fetal subgroup. Moreover, 31.6% of the SNPs that were analyzed in the black fetal subgroup were also found to be out of HWE. The mismatch of original fetal sample genotypes and new fetal samples genotypes in both subgroups also suggests that the DNA sample quality is an issue particularly when using the iPLEX® platform, or there may have been aliquotting issues or sample mix ups that occurred at some point ranging from sample collection to genotype data collection. Given these findings, the

decision was made to not proceed with the analysis of aim 2 in both the white subgroup and black subgroup.

2.3 STUDY STRENGTHS AND LIMITATIONS

There were several strengths associated with this dissertation study. The major strength of this project is that study of the *ENG* pathway at the molecular level is novel. The pathway approach, rather than a singular gene approach, also allows one to draw global and biologically meaningful conclusions, resulting in increased understanding of preeclampsia pathophysiology. The tSNP approach also gave us the ability to fully evaluate the genetic variability of each candidate gene with the fewest number of SNPs.

There were also several limitations associated with this dissertation study. This project was limited to and guided by the data/samples collected by the parent study. This limitation was most evident in Aim 2. Because fetal DNA extracted from white blood cell pellets was not available, we used the available umbilical cord serum samples for DNA extraction. Unfortunately, we found that this template was not suitable for genotype data collection with iPLEX® and we were not able to analyze Aim 2. The small black subgroup sample size, which could lead to type 2 error, and the use of tSNPs only selected for Caucasian ancestry, which may not guarantee sufficient coverage of the candidate genes in the Black subgroup, represent two additional limitations.

2.4 FUTURE STUDIES AND IMPLICATIONS FOR GLOBAL HEALTH AND NURSING

Preeclampsia is a global problem that affects women of all ancestries. Future studies are needed to confirm the results of this study in different samples of women from non-Caucasian ancestries. Moreover, studies examining *ENG* pathway genetic variation in women of other ancestries are needed to determine if *ENG* pathway variation is universally involved in preeclampsia development regardless of ancestry. Because our results suggest that the *ENG* pathway genes involved in preeclampsia differ in white and black women, studies examining how these documented variations impact the function of *ENG* pathway candidate genes will further improve our understanding of how preeclampsia etiology may differ in women of different ancestries. Confirmation of these results and exploration of the functional significance associated with these variations could lead to the development of clinically relevant, stable biomarkers of preeclampsia susceptibility that can be used in the early identification of at risk women around the globe. Ultimately, as frontline providers, nurses are likely to be the healthcare providers that would be involved in the education of patients on biomarker testing, the administration of biomarker testing, and the creation of individualized nursing care plans based on biomarker test results.

3.0 DATA-BASED MANUSCRIPT: VARIATION IN ENDOGLIN PATHWAY GENES IS ASSOCIATED WITH PREECLAMPSIA

3.1 ABSTRACT

This case-control candidate gene association study investigated endoglin (*ENG*) pathway genetic variation and its association with preeclampsia. Data on 355 white women (181 cases/174 controls) and 60 black women (30 cases/30 controls) matched on ancestry, age, and parity were analyzed. Tagging single nucleotide polymorphisms (tSNPs) in *ENG*, *TGFβ1*, *TGFβR1*, *ALK1*, and *TGFβR2* were evaluated with iPLEX® and TaqMan® technologies. Allele/genotype/haplotype tests were conducted separately in white/black subgroups with a $\chi 2$ test or Fisher's exact test. Odds ratios were computed with binary logistic regression for tSNPs with significant genotype tests. In white women, variation in *ENG* (rs11792480, rs10121110) and *TGFβR2* (rs6550005) was associated with preeclampsia. Allelic frequency distributions in rs11792480, rs10121110, and rs6550005 were significantly different among cases and controls while genotype distributions of rs10121110 and rs6550005 were further associated with preeclampsia (p-values < .05). For rs10121110, women with the AA genotype were 2.290 times more likely to develop preeclampsia compared to the GG genotype (99% CI [1.022, 5.133], p = .008). The *ENG* haplotype TACGA, which contains the risk alleles for rs11792480 and rs10121110, was also over-represented in cases (p = .022). In black women, variation in *TGFβ1* (rs4803455, rs4803457), *TGFβR1* (rs10739778), and *TGFβR2* (rs6550005, rs1346907, rs877572) was associated with preeclampsia. Allelic frequency distributions in rs10739778, rs6550005, rs1346907, and rs877572 were significantly different among cases and controls while

genotype distributions of rs10739778, rs4803455, and rs4803457 were further associated with preeclampsia (p-values < .05). For rs4803457, women with the CT genotype were 7.437 more times likely to develop preeclampsia compared to the CC genotype (99% CI [1.192, 46.408], p = .005). These results demonstrate that variation in *ENG* pathway genes is associated with preeclampsia in white and black women, with different genes from the same pathway being involved in white women compared to black women.

Keywords: preeclampsia, endoglin pathway, tagging SNPs, iPLEX®

3.2 INTRODUCTION

Preeclampsia is a multi-system disorder of pregnancy that is clinically diagnosed when a previously normotensive women presents with new onset hypertension and proteinuria after 20 weeks' gestation (American College of Obstetricians and Gynecologists [ACOG], 2002; National Heart, Lung, and Blood Institute [NHLBI] National High Blood Pressure Education Program, 2000). Although we have made great strides in trying to identify preeclampsia's pathophysiology, its heterogeneous nature suggests that a variety of mechanisms rather than a singular, universal mechanism may lead to preeclampsia. Research focusing on the role of the anti-angiogenic factor endoglin (ENG) in preeclampsia has identified one such mechanism that may contribute to the development of preeclampsia in a subgroup of women.

ENG is a trans-membrane glycoprotein that serves as a co-receptor of the transforming growth factor beta (TGFβ) signaling system (Cheifetz et al., 1992). It is expressed on vascular endothelial cells (Gougos & Letarte, 1990), synctyiotrophoblasts, and transitioning

cytotrophoblasts (St-Jacques, Forte, Lye, & Letarte, 1994). ENG is also involved in the maintenance of vascular tone by regulating nitric oxide dependent vasodilatation (Jerkic et al., 2004; Toporsian et al., 2005). Moreover, ENG is likely to be involved in the regulation of placental implantation and spiral artery remodeling during pregnancy (Caniggia, Taylor, Ritchie, Lye, & Letarte, 1997; Mano et al., 2011). Both Caniggia, Taylor, Know Ritchie, Lye and Letarte (1997) and Mano et al. (2011) have demonstrated that inhibition of *ENG* translation, either in first trimester human villous explants or a human extravillous tropholast (EVT) cell line, has improved the invasive capacity of EVTs.

Given that systemic endothelial dysfunction and shallow placental implantation/spiral artery remodeling are hallmark abnormalities observed in preeclampsia (Roberts & Hubel, 2009), investigation of ENG's potential role has been warranted and the results thus far are promising. To date, multiple studies have found that *ENG* gene expression (mRNA) is increased in the placenta and/or blood throughout pregnancy in women who develop preeclampsia (Farina et al., 2008; Farina et al., 2010; Nishizawa et al., 2007; Purwosunu et al., 2008; Purwosunu, Sekizawa, Okazaki, et al., 2009; Purwosunu, Sekizawa, Yoshimira, et al., 2009; Sekizawa et al., 2012; Sitras et al., 2009; Toft et al., 2008; Tsai et al., 2011; Venkatesha et al., 2006). Soluble endoglin (sENG) protein, which is released into circulation after cleavage of trans-membrane ENG by MMP-14 (Kaitu'u-Lino et al., 2012), has also been found to be elevated in preeclampsia compared to healthy controls (Rana et al., 2007). Despite these findings, little research has examined what contributes to the differences in *ENG* mRNA expression and sENG protein levels in women with preeclampsia. The purpose of this study was to investigate how variation in endoglin pathway genes (*ENG*, *TGFβ1*, *TGFβR1*, *ALK1*, and *TGFβR2*) may be associated with the development of preeclampsia. Tagging single nucleotide

polymorphisms (tSNPs) and potentially functional SNPs identified in the literature were used to evaluate candidate gene variability in women with preeclampsia compared to healthy controls that were matched on age (+/- 2 years), ancestry (white/black), and parity.

3.3 METHODS

3.3.1 Study population

This case-control, candidate gene association study obtained subjects with and without preeclampsia from the first two cohorts of the Prenatal Exposures and Preeclampsia Prevention (PEPP) study. Conducted at Magee-Womens Hospital of UPMC (Pittsburgh, Pa), the PEPP study examines factors predisposing women to preeclampsia via two recruitment approaches. In PEPP's first two cohorts, women 14-44 years of age were recruited/enrolled during early pregnancy (≤ 20 weeks' gestation) and followed through delivery/postpartum period at Magee-Womens Hospital or they were recruited/enrolled at the labor/delivery unit of Magee-Womens Hospital due to a suspected diagnosis of preeclampsia. Women with a history of chronic renal disease, hypertension, diabetes, or other disorders that increased the risk of preeclampsia were excluded from longitudinal enrollment. Cross-sectionally enrolled women found to have the aforementioned conditions were excluded from any analyses involving women with preeclampsia and healthy controls. Demographic, clinical, and laboratory data were collected by appropriately trained individuals. Genomic DNA was extracted from peripherally collected venous blood samples via protein precipitation from white blood cell pellets. All aspects of

PEPP, including the use of samples/data for genetically-based research, were approved by the University of Pittsburgh and Magee Womens Hospital Institutional Review Board (IRB). Separate University of Pittsburgh IRB approval was also granted for this study. PEPP subjects not consenting to future genetic evaluation and subjects without a stored genetic sample were excluded from this study.

3.3.2 Phenotype classification

Determination of pregnancy outcome was made after the review of clinical data by an expert panel of clinicians/researchers. Preeclampsia diagnosis was based on blood pressure (BP), urinary protein, and serum uric acid criteria. The average of the last five BPs taken in the hospital prior to therapeutic intervention (e.g., medication, anesthesia) were compared to the average BP prior to 20 weeks' gestation to establish the presence/absence of hypertension. Hypertension was defined as a BP $\geq$ 140 mmHg systolic and/or 90 mmHg diastolic AND an increase of BP > 30 mmHg systolic and/or 15 mmHg diastolic after 20 weeks' gestation. Blood pressure abnormalities were to resolve by 12 weeks postpartum. Proteinuria was defined as $\geq$ 300 mg/ 24hours, $\geq$ 0.3 protein/creatinine ratio, $\geq$ 2+ on a random urine specimen, or $\geq$ 1+ on a catheterized urine specimen. Hyperuricemia was defined as a serum uric acid concentration > 1 standard deviation from normal for gestational age (Lind, Godfrey, Otun, & Philips, 1984). A severe preeclampsia diagnosis was made when subjects with preeclampsia also had $\geq$ 1 of the following conditions: (a) systolic BP $\geq$ 160 mmHg, (b) diastolic BP $\geq$ 110 mmHg, (c) proteinuria $\geq$ 5 grams/ 24 hours, (d) elevated liver enzymes, or (e) platelet count $\leq$ 100,000. Hemolysis,

elevated liver enzymes, and low platelets in subjects with preeclampsia indicated the presence of HELLP syndrome. Clinically evaluated subjects that had negative past medical histories (e.g., chronic renal disease, hypertension, diabetes) and did not meet the criteria for preeclampsia, severe preeclampsia, or HELLP syndrome were designated as healthy controls.

For this case-control study, the case group included PEPP subjects diagnosed with preeclampsia, severe preeclampsia, or HELLP syndrome while the control group was comprised of healthy PEPP subjects. A total of 215 controls were 1:1 frequency matched to 215 cases on self-reported ancestry (black/white), age, and parity.

3.3.3 Polymorphism selection

To fully evaluate the genetic variability of the candidate genes (*ENG*, *TGFβ1*, *TGFβR1*, *ALK1*, and *TGFβR2*), including upstream and downstream regulatory regions, tSNPs were selected from the International HapMap Project database (HapMap Data Phase III/Rel#2, Feb09, on NCBI B36 assembly, dbSNP b126). tSNP selection was based on the following criteria: (a) minor allele frequency $\geq$ 20% for each SNP, (b) R^2 cutoff= 0.8, and (c) Caucasian (CEU) ancestry given that the majority of the sample self classified as white. Potentially functional SNPs identified in the scientific literature were also included in the assessment of the candidate genes. A total of 47 tSNPs and 2 potentially functional SNPs were selected for evaluation (Table 6). The UCSC Genome Browser (Fujita et al., 2011) was utilized to identify the nucleotide position of the selected SNPs. The UCSC Genome Browser (Fujita et al.,) or the Database of Single Nucleotide Polymorphisms (dbSNP; http://www.ncbi.nlm.nih.gov/projects/SNP/) was used to identify the location of the significantly associated tSNPs within the genes of interest (e.g., exon, intron).

Table 6. iPLEX Assays

Assay 1[a]		Assay 2	
Gene	rs numbers	Gene	rs numbers
ENG	rs10987746, rs10819309, rs10760505, rs11792480, rs10121110	*TGFβR2*	rs2043136, rs13075948, rs1346907, rs3773652, rs4955212, rs1155708, rs3773640, rs1036097, rs2082224, rs876688, rs744751, rs1078985, rs17025785, rs877572, rs5020833, rs6809777, rs6802220, rs9843942, rs6550005, rs3773644, rs3773645, rs13083813, rs12487185, rs13086588, rs6792117, rs3773663, rs4522809, rs11129420, rs995435, rs11924422
TGFβ1	rs4803455, rs1800469, rs4803457, rs8179181, rs1800468, rs11466314		
TGFβR1	rs6478974, rs420549, rs10739778		
ALK1	rs3759178, rs11169953, rs706819		
TGFβR2	rs749794, rs3087465		

Note. [a]Additional space in Assay 1 allowed for inclusion of 12 non-candidate gene tSNPs not analyzed within the context of this project. rs# for these tSNPs are not shown.

3.3.4 Genotyping methods

The iPLEX® Gold-SNP Genotyping assay (Sequenom® Inc, San Diego, CA) was utilized to genotype the 49 SNPs in each of the 430 subjects. Two multiplex assays consisting of two amplification primers and one single base extension primer for each SNP locus were designed with the online Human GenoTyping Tools and MassARRAY® Designer v4.0 software (https://www.mysequenom.com). Genotyping was conducted at the University of Pittsburgh Genomics and Proteomics Core Labratories using the MassARRAY® Nanodispenser (Samsung, Irvine, CA), MassARRAY® Compact Analyzer (Bruker, Newark, DE), SpectroACQUIRE v3.3.1.3 software (Sequenom®), and MassARRAY® Typer v4.0 software (Sequenom®) (http://www.genetics.pitt.edu/).

Five SNPs did not meet data quality criteria with the iPLEX® platform. Three SNPs (rs1800468, rs10739778, rs6809777) had genotype call rates of < 86% and two SNPs

(rs3087465, rs8179181) were multi-allelic (> 2 alleles), causing genotype data validity to be questioned. For these five SNPs, we performed TaqMan® allelic discrimination with the ABI Prism® 7000 Sequence Detection System and SDS software v1.2.3 (Applied Biosystems Inc., Carlsbad, CA), using pre-developed and commercially available TaqMan® SNP Genotyping assays (Applied Biosystems®). We used the following thermal profile: (a) AmpliTaq Gold Enzyme activation at 95°C for 10 minutes, (b) denature at 95°C for 15 seconds, (c) anneal/extend at 58°C for 1:30 minute, (d) go to step b 50 times, (e) hold at 10°C. Genotype data collection for these five SNPs was collected at the University of Pittsburgh School of Nursing Molecular Genetics Laboratory.

3.3.5 Genotype data reliability, haplotype assignment, and linkage disequilibrium estimation

Reliability of genotype data was evaluated in several steps, with attention focused on validation of data for consistency and integrity. Steps included comparison of expected homozygosity to observed homozygosity at each SNP, comparison of study allele frequencies to allele frequencies from the Database of Short Genetic Variations (dbSNP) (http://www.ncbi.nlm.nih.gov/snp), consideration of genotype call rates, inclusion of blind duplicates, double call of genotypes, and checks for Hardy-Weinberg Equilibrium (HWE) consistency. HWE calculations were conducted using PLINK software version 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007) or the online HWE calculator available at http://www.oege.org/software/hwe-mr-calc.shtml. In both the non-related white and black subgroups, PLINK software was used to estimate *ENG* haplotypes. Pair-wise linkage disequilibrium (R^2 and D') across the *ENG* gene

was also estimated in the white subgroup. Due to small sample size and haplotype frequencies, we did not conduct further haplotype analysis in the black subgroup.

3.3.6 Statistical analysis

Within each ancestral subgroup (white, black), demographic characteristics were compared between cases and controls. Continuous variables with parametric distributions were assessed with either an independent samples t-test or an independent samples t-test with unequal variances. The Mann-Whitney U test was used to assess continuous variables with non-parametric distributions. Categorical variables were assessed with either the Mann-Whitney U test or the $\chi 2$ test of independence. Multiple imputation was utilized to estimate missing pre-pregnancy body mass index (BMI) values for $n = 22$ white cases.

For both ancestral subgroups, a $\chi 2$ goodness-of-fit test or an exact test was used to detect deviations from HWE. tSNPs and/or functional SNPs that violated HWE ($p < .005$) were further assessed by checking for HWE consistency in cases and controls separately.

Chi-square tests of independence were used to test the association between the candidate gene tSNP/functional SNP alleles and preeclampsia status (allele test) in each ancestral subgroup. The association between tSNP/functional SNP genotypes and preeclampsia status (genotype test) in each ancestral subgroup was tested with either a $\chi 2$ test of independence or a Fisher's exact test. SNPs with homozygote variant frequencies of < 10% in either cases, controls, or both were dichotomized (homozygote wildtype; homozygote variant + heterozygote) prior to conducting the genotype test. Binary logistic regression was utilized to compute odds ratios and 99% confidence intervals (CI) for SNPs found to have a significant genotype test. A

99% CI was selected to account for inflation of type 1 error resulting from multiple testing. As an effect size statistic, the odds ratio provided information on the direction and magnitude of the relationship (Lipsey & Wilson, 2001) between genotype assignment and preeclampsia status. Odds ratio values of 2.0, 3.0, and 4.0 were considered small, moderate, and strong effects, respectively (Ferguson, 2009).

The association between genotype assignment and clinical characteristics (blood pressure, pre-pregnancy BMI) was also assessed in SNPs with significant genotype tests. An ANOVA, independent samples t-test, Kruskal Wallis test, or Mann-Whitney U test was used to test these associations according to number of genotype groups and sample distributions. Statistical analyses were conducted with SPSS version 19 (SPSS Inc., Chicago, IL.).

The most probable *ENG* haplotypes estimated for each white subject were selected for analysis. Haplotype allele frequencies were analyzed separately in cases and controls. Haplotypes with < 10 % in either cases, controls, or both were collapsed into one category. A $\chi 2$ test of independence was used to determine if the frequency distributions of the 4 haplotype categories differed in cases and controls. Separate pair-wise comparisons of haplotype frequency distributions were also analyzed. Diplotypes were also generated from the *ENG* haplotypes. Diplotypes were generated based on the categories formed during the haplotype test. Diplotypes with frequencies < 10% in either cases, controls, or both were combined into one category. SPSS version 19 (SPSS Inc., Chicago, IL.) was used to compare the association between diplotype and preeclampsia status via a $\chi 2$ test of independence.

3.4 RESULTS

3.4.1 White subgroup

3.4.1.1 Demographic and clinical characteristics

Demographic and clinical characteristics for the white subgroup comprised of 181 cases and 174 controls (see Figure 2 for final sample size explanation) are presented in Table 7. A post hoc power analysis using Quanto version 1.2.4 revealed that a sample of 181 controls matched to 181 cases resulted in a power ranging from .8979 to .9990 for an $\alpha = .05$ and .07437 to .9934 for an $\alpha = .01$. Cases and controls did not significantly differ for variables on which they were matched (age, parity). Women with preeclampsia had a higher pre-pregnancy BMI (M = 25.8 kg/m^2 vs. M = *22*.9 kg/m^2, $p < .001$), a higher average blood pressure at < 20 weeks gestation (SBP: M = 116.6 mmHg vs. M = 112.1 mmHg, $p < .001$; DBP: M = 71.7 mmHg vs. M = 68.1 mmHg, $p < .001$) and a higher average blood pressure in labor (SBP: M = 154.8 mmHg vs. M = 120.4 mmHg, $p < .001$; DBP: M = 92.6 mmHg vs. M = 72.3 mmHg, $p < .001$) compared to healthy controls. Gestational age at delivery was earlier in women with preeclampsia (M = 36.1 weeks vs. M = 39.6 weeks, $p < .001$) and the percentage of cesarean section deliveries was higher in women with preeclampsia (42% vs. 18.1%, $p < .001$). Babies born to women with preeclampsia were of lower birthweight (M = 2497.5 grams vs. M = 3481.6 grams, $p < .001$) and were more likely to be small for gestational age (26.0% vs. 4.0%, $p < .001$).

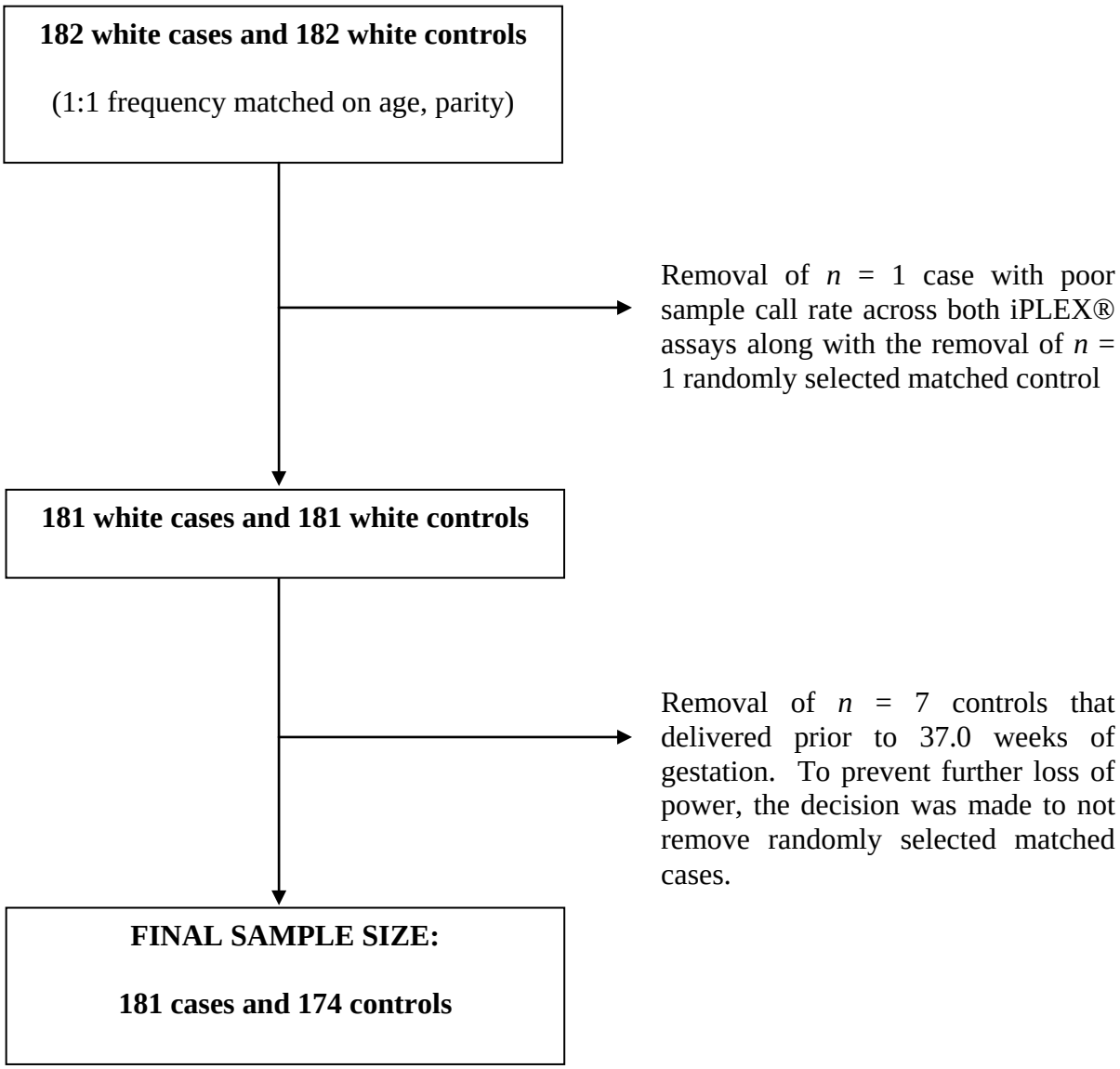


Figure 2. Sample Flow Chart of White Subgroup

Table 7. Demographic and Clinical Characteristics of White Subgroup

Variable	Cases (*n* = 181)	Controls (*n* = 174)	p-value
Maternal age (*M (SD)*, years)	28.3 (5.8)	28.4 (5.7)	.866[a]
Gravida (*Mdn (min-max)*)	1 (1-6)	1 (1-8)	.082[b]
Nulliparous (*n, %)*	146 (80.7%)	139 (79.9%)	.854[c]
Gestational age at delivery (*M (SD)*, weeks)	36.1 (3.2)	39.6 (1.1)	**< .001**[d]
Birthweight (*M (SD)*, grams)[e]	2497.5 (841.2)	3481.6 (446.3)	**< .001**[d]
Caesarean section (*n, %*)[f]	74 (42%)	30 (18.1%)	**< .001**[c]
Small for gestational age (*n, %*)	47 (26.0%)	7 (4.0%)	**< .001**[c]
Avg. SBP < 20 weeks gestation (*M (SD)*, mmHg)[g]	116.6 (9.6)	112.1 (7.5)	**< .001**[d]
Avg. DBP < 20 weeks gestation (*M (SD)*, mmHg)[g]	71.7 (7.2)	68.1 (4.9)	**< .001**[d]
Avg. SBP in labor (*M (SD)*, mmHg)[h]	154.8 (13.9)	120.4 (10.2)	**< .001**[d]
Avg. DBP in labor (*M (SD)*, mmHg)[i]	92.6 (8.0)	72.3 (7.2)	**<. 001**[a]
Pre-pregnancy BMI (*Mdn (min-max)*)[j]	25.8 (17-46)	22.9 (16-37)	**< .001**[b]

Note. [a]Independent samples t-test; [b]Mann-Whitney U test; [c]$\chi 2$ test of independence; [d]Independent samples t-test with unequal variances; [e]Outlier removed in control group; [f]*n* = 176 cases and *n* = 166 controls due to missing data; Avg. = average; SBP = systolic blood pressure; mmHg = millimeters of mercury; [g]*n* = 168 cases and *n* = 170 controls due to missing data; DBP = diastolic blood pressure; [h]*n* = 181 cases and *n* = 173 controls due to missing data; [i]*n* = 180 cases and *n* = 173 controls due to missing data and removal of outlier in case group; BMI = body mass index; [j]BMI values imputed for *n* = 22 cases, sample size was *n* = 178 cases and *n* = 172 controls due to removal of outliers

3.4.1.2 Genotype call rates, MAF, HWE

Descriptive information for each tSNP and functional SNP is provided in Table 8. tSNPs rs8179181 and rs3087465 could not be genotyped, despite multiple attempts with iPLEX® and TaqMan®, and functional SNP rs11466314 was monomorphic in the white subgroup, resulting in their omission from analysis. The remaining 46 SNPs had genotype call rates that ranged

from 98% – 100%. Of the 46 SNPs included in the analysis, five tSNPs violated HWE ($p < .05$) in the white subgroup. One tSNP was located in *TGFβR1* (rs10739778), one tSNP was located in *ENG* (rs11792480), and three tSNPs were located in *TGFβR2* (rs3773652, rs1346907, rs877572). Separate evaluation of HWE in cases and controls revealed that rs10739778 was in HWE in controls ($p = .846$), rs11792480 was in HWE in cases ($p = .193$), rs3773652 was in HWE in controls and cases separately ($p = .069$ & $p = .238$), rs1346907 was in HWE in controls ($p = .098$), and rs877572 was in HWE in controls ($p = .315$).

Table 8. tSNP and Functional SNP Information in White Subgroup (*N* = 355)

Gene-Chromosome tSNP	Wildtype Allele/ Nucleotide #/ Variant Allele[a]	*n for each SNP*	Study MAF	HapMap MAF	HWE[b]
ALK5(TGFBR1)-Chr9					
rs6478974	T/101874403/A	349	A = .483	A = .469	p = .434
rs10739778	A/101875789/C	352	C = .314	C = .292	**p = .040**
rs420549	G/101914873/C	350	C = .169	C = .204	p = .433
ALK1-Chr12					
rs3759178	T/52299259/G	350	G = .369	G = .398	p = .917
rs11169953	C/52304399/T	350	T = .351	T = .250	p = .958
rs706819	G/52315923/A	350	A = .226	A = .286	p = .799
TGFB1-Chr19					
rs8179181	--/41838206/--	----	----	----	----
rs4803455	C/41851509/A	350	A = .487	A = .496	p = .840
rs11466314	G/41860236/A	350	A = .000	A = .000	----
rs1800469	C/41860296/T	350	T = .301	T = .288	p = .286
rs1800468	C/41860587/T	353	T = .086	T = .050	p = .312
rs4803457	C/41861359/T	350	T = .384	T = .381	p = .601
ENG-Chr9					
rs10987746	T/130580093/C	349	C = .493	C = .415	p = .869
rs10819309	G/130581723/A	349	A =.367	A = .473	p = .99
rs10760505	C/130589853/T	348	T = .386	T = .341	p = .495
rs11792480	G/130598125/A	350	A = .326	A = .353	**p = .008**
rs10121110	A/130602408/G	348	G = .392	G = .412	p = .057
TGFBR2-Chr3					
rs3087465	--/30647160/--	----	----	----	----
rs6550005	G/30650064/A	355	A = .192	A = .243	p = .088
rs11129420	A/30658541/T	355	T = .475	A = .487	p = .671
rs6802220	G/30659652/A	355	A = .414	A = .363	p = .199

Gene-Chromosome tSNP	Wildtype Allele/ Nucleotide #/ Variant Allele[a]	*n for each SNP*	Study MAF	HapMap MAF	HWE[b]
rs17025785	T/30667425/C	355	C = .334	C = .330	p = .708
rs4522809	C/30668684/T	355	T = .499	C = .478	p = .708
rs4955212	C/30669358/T	351	T = .268	T = .252	p = .752
rs5020833	C/30670425/G	355	G = .304	G = .296	p = .823
rs6809777	C/30672362/T	355	T = .261	T = .270	p = .104
rs12487185	A/30677269/G	355	G = .283	G = .323	p = .146
rs11924422	A/30677484/C	355	C = .431	C = .456	p = .655
rs13083813	T/30679558/A	354	A = .377	A = .398	p = .888
rs13075948	C/30683506/T	355	T = .275	T = .265	p > .999
rs1155708	G/30686740/A	354	A = .322	A = .336	p = .424
rs13086588	T/30688757/G	355	G = .314	G = .332	p = .141
rs2082224	G/30689755/A	355	A = .235	A = .235	p = .920
rs1078985	T/30690911/C	349	C = .256	C = .332	p = .764
rs1036097	G/30693643/A	355	A = .472	A = .412	p = .671
rs995435	C/30700922/T	348	T = .263	T = .257	p = .791
rs6792117	A/30704007/G	353	G = .482	A = .451	p = .689
rs749794	T/30708432/C	350	C = .319	C = .332	p = .532
rs3773640	A/30709511/T	355	T = .242	T = .270	p > .999
rs3773644	C/30712344/T	355	T = .380	T = .438	p = .450
rs3773645	C/30712460/G	354	G = .301	G = .367	p = .308
rs3773652	A/30718942/G	355	G = .480	G = .469	**p = .032**
rs2043136	T/30720304/C	355	C = .268	C = .239	p = .484
rs1346907	C/30723470/T	355	T = .473	T = .451	**p = .001**
rs876688	G/30725776/A	354	A = .340	A = .376	p = .238
rs877572	G/30726432/C	355	C = .466	C = .460	**p = .021**
rs9843942	G/30729636/A	354	A = .380	A = .375	p = .842
rs3773663	G/30730872/A	355	A = .396	A = .429	p = .752
rs744751	C/30735937/T	355	T = .265	T = .363	p = .396

Note. MAF = mean allele frequency; HWE= hardy weinberg equilibrium; [a]wildtype and variant alleles based on study sample, nucleotide # obtained from UCSC Genome Browser assembly Feb. 2009 (GRCh37/hg19) (Fujita et al., 2011); [b] $\chi 2$ goodness-of-fit test or exact test (significant results bolded); ---- = not analyzed

3.4.1.3 Allele test

Allele test results are presented in Table 9. In *ENG*, allelic frequency distributions for rs11792480 and rs10121110 were significantly different in cases and controls (Figure 3a-b). Compared to controls, the G allele of rs11792480 was over-represented in cases (71.7% vs. 63.0%, p = .014) and the A allele of rs10121110 was over-represented in cases (66.0% vs.

55.3%, p = .004). In *TGFβR2*, the allelic frequency distribution for rs6550005 was significantly different in cases and controls (Figure 3c). Compared to controls, the G allele of rs6550005 was over-represented in cases (84.0% vs. 77.6%, p = .031). Allele tests for the remaining tSNPs and functional SNP demonstrated no significant differences.

Table 9. Results of Association Analysis in White Subgroup (N = 355)

Gene SNP	Allele Counts (%) Cases	Allele Counts (%) Controls	Total Alleles (*n*)	Allele Test[a]	Genotype Counts (%) Cases	Genotype Counts (%) Controls	Total Genotypes (*n*)	Genotype Test[b]
ALK5 (TGFBR1)								
rs6478974	T: 185 (51.7%) A: 173(48.3%)	T: 176 (51.8%) A: 164 (48.2%)	698	p > .999	TT: 50 (27.9%) AA: 44 (24.6%) TA: 85 (47.5%)	TT: 47 (27.6%) AA: 41 (24.1%) TA: 82 (48.2%)	349	p = .990
rs10739778*	A: 244 (68.2%) C: 114 (31.8%)	A: 239 (69.1%) C: 107 (30.9%)	704	p = .791	AA: 92 (51.4%) CC: 27 (15.1%) AC: 60 (33.5%)	AA: 82 (47.4%) CC: 16 (9.2%) AC: 75 (43.4%)	352	p = .453
rs420549*	G: 296 (82.2%) C: 64 (17.8%)	G: 286 (84.1%) C: 54 (15.9%)	700	p = .502	GG: 125 (69.4%) CC: 9 (5.0%) GC: 46 (25.6%)	GG: 119 (70.0%) CC: 3 (1.8%) GC: 48 (28.2%)	350	p = .910
ALK1								
rs3759178	T: 235 (65.3%) G: 125 (34.7%)	T: 207 (60.9%) G: 133 (39.1%)	700	p = .229	TT: 75 (41.7%) GG: 20 (11.1%) GT: 85 (47.2%)	TT: 65 (38.2%) GG: 28 (16.5%) GT: 77 (45.3%)	350	p = .340
rs11169953	C: 228 (63.3%) T: 132 (36.7%)	C: 226 (66.5%) T: 114 (33.5%)	700	p = .383	CC: 72 (40.0%) TT: 24 (13.3%) CT: 84 (46.7%)	CC: 75 (44.1%) TT: 19 (11.2%) CT: 76 (44.7%)	350	p = .685
rs706819*	G: 282 (78.3%) A: 78 (21.7%)	G: 260 (76.5%) A: 80 (23.5%)	700	p = .554	GG: 111 (61.7%) AA: 9 (5.0%) GA: 60 (33.3%)	GG: 98 (57.6%) AA: 8 (4.7%) GA: 64 (37.6%)	350	p = .444
TGFB1								
rs4803455	C: 176 (48.9%) A: 184 (51.1%)	C: 183 (53.8%) A: 157 (46.2%)	700	p = .192	CC: 41 (22.8%) AA: 45 (25.0%) CA: 94 (52.2%)	CC: 52 (30.6%) AA: 39 (22.9%) CA: 79 (46.5%)	350	p = .253
rs1800469*	C: 259 (71.9%) T: 101 (28.1%)	C: 230 (67.6%) T: 110 (32.4%)	700	p = .216	CC: 94 (52.2%) TT: 15 (8.3%) CT: 71 (48.3%)	CC: 81 (47.6%) TT: 21 (12.4%) CT: 68 (40.0%)	350	p = .392

Gene SNP	Allele Counts (%) Cases	Allele Counts (%) Controls	Total Alleles (*n*)	Allele Test[a]	Genotype Counts (%) Cases	Genotype Counts (%) Controls	Total Genotypes (*n*)	Genotype Test[b]
rs1800468*	C: 325 (90.8%) T: 33 (9.2%)	C: 320 (92.0%) T: 28 (8.0%)	706	p = .578	CC: 147 (82.1%) TT: 1 (.6%) CT: 31 (17.3%)	CC: 149 (85.6%) TT: 3 (1.7%) CT: 22 (12.6%)	353	p = .370
rs4803457	C: 229 (63.6%) T: 131 (36.4%)	C: 202 (59.4%) T: 138 (40.6%)	700	p = .254	CC: 71 (39.4%) TT: 22 (12.2%) CT: 87 (48.3%)	CC: 64 (37.6%) TT: 32 (18.8%) CT: 74 (43.5%)	350	p = .225
ENG								
rs10987746	T: 192 (53.6%) C: 166 (46.4%)	T: 162 (47.6%) C: 178 (52.4%)	698	p = .114	TT: 51 (28.5%) CC: 38 (21.2%) TC: 90 (50.3%)	TT: 38 (22.4%) CC: 46 (27.1%) TC: 86 (50.6%)	349	p = .283
rs10819309	G: 219 (61.2%) A: 139 (38.8%)	G: 223 (65.6%) A: 117 (34.4%)	698	p = .227	GG: 65 (36.3%) AA: 25 (14.0%) GA: 89 (49.7%)	GG: 75 (44.1%) AA: 22 (12.9%) GA: 73 (42.9%)	349	p = .324
rs10760505	C: 222 (62.0%) T: 136 (38.0%)	C: 205 (60.7%) T: 133 (39.3%)	696	p = .708	CC: 70 (39.1%) TT: 27 (15.1%) CT: 82 (45.8%)	CC: 64 (37.9%) TT: 28 (16.6%) CT: 77 (45.6%)	348	p = .925
rs11792480	G: 258 (71.7%) A: 102 (28.3%)	G: 214 (63.0%) A: 126 (37.0%)	700	**p = .014**	GG: 96 (53.3%) AA: 18 (10.0%) AG: 66 (36.7%)	GG: 74 (43.5%) AA: 30 (17.6%) AG: 66 (38.8%)	350	p = .062
rs10121110	A: 235 (66.0%) G: 121 (34.0%)	A: 188 (55.3%) G: 152 (44.7%)	696	**p = .004**	AA: 81 (45.5%) GG: 24 (13.5%) AG: 73 (41.0%)	AA: 56 (32.9%) GG: 38 (22.4%) AG: 76 (44.7%)	348	**p = .022**
TGFBR2								
rs6550005*	G: 304 (84.0%) A: 58 (16.0%)	G: 270 (77.6%) A: 78 (22.4%)	710	**p = .031**	GG: 130 (71.8%) AA: 7 (3.9%) GA: 44 (24.3%)	GG: 107 (61.5%) AA: 11 (6.3%) GA: 56 (32.2%)	355	**p = .039**
rs11129420	A: 193 (53.3%) T: 169 (46.7%)	A: 180 (51.7%) T: 168 (48.3%)	710	p = .671	AA: 50 (27.6%) TT: 38 (21.0%) TA: 93 (51.4%)	AA: 46 (26.4%) TT: 40 (23.0%) TA: 88 (50.6%)	355	p = .897

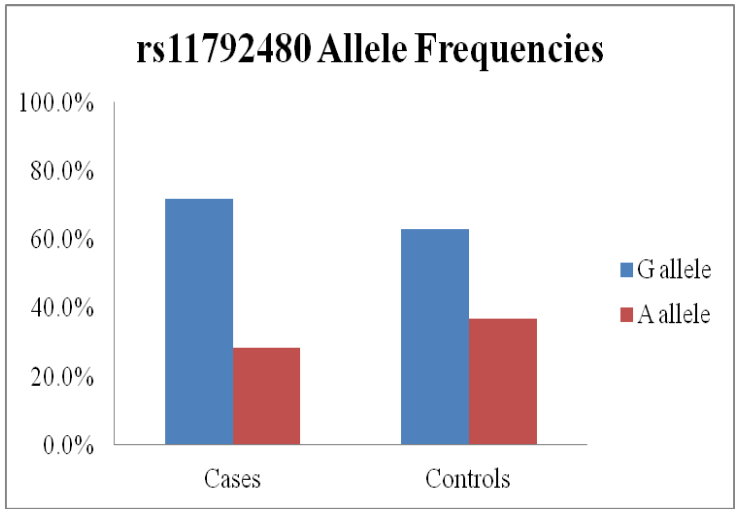
Gene SNP	Allele Counts (%) Cases	Allele Counts (%) Controls	Total Alleles (*n*)	Allele Test[a]	Genotype Counts (%) Cases	Genotype Counts (%) Controls	Total Genotypes (*n*)	Genotype Test[b]
rs6802220	G: 216 (59.7%) A: 146 (40.3%)	G: 200 (57.5%) A: 148 (42.5%)	710	p = .554	GG: 64 (35.4%) AA: 29 (16.0%) AG: 88 (48.6%)	GG: 52 (29.9%) AA: 26 (14.9%) AG: 96 (55.2%)	355	p = .446
rs17025785	T: 240 (66.3%) C: 122 (33.7%)	T: 233 (67.0%) C: 115 (33.0%)	710	p = .862	TT: 79 (43.6%) CC: 20 (11.0%) TC: 82 (45.3%)	TT: 77 (44.3%) CC: 18 (10.3%) TC: 79 (45.4%)	355	p = .976
rs4522809	C: 185 (51.1%) T: 177 (48.9%)	C: 171 (49.1%) T: 177 (50.9%)	710	p = .603	CC: 48 (26.5%) TT: 44 (24.3%) CT: 89 (49.2%)	CC: 43 (24.7%) TT: 46 (26.4%) CT: 85 (48.8%)	355	p = .872
rs4955212*	C: 257 (71.8%) T: 101 (28.2%)	C: 257 (74.7%) T: 87 (25.3%)	702	p = .383	CC: 91 (50.8%) TT: 13 (7.3%) CT: 75 (41.9%)	CC: 96 (55.8%) TT: 11 (6.4%) CT: 65 (37.8%)	351	p = .350
rs5020833*	C: 252 (69.6%) G: 110 (30.4%)	C: 242 (69.5%) G: 106 (30.5%)	710	p > .999	CC: 88 (48.6%) GG: 17 (9.4%) CG: 76 (42.0%)	CC: 83 (47.7%) GG: 15 (8.6%) CG: 76 (43.7%)	355	p = .863
rs6809777*	C: 270 (74.6%) T: 92 (25.4%)	C: 255 (73.3%) T: 93 (26.7%)	710	p = .689	CC: 103 (56.9%) TT: 14 (7.7%) CT: 64 (35.4%)	CC: 97 (55.7%) TT: 16 (9.2%) CT: 61 (35.1%)	355	p = .826
rs12487185*	A: 261 (72.1%) G: 101 (27.9%)	A: 248 (71.3%) G: 101 (28.7%)	710	p = .806	AA: 101 (55.8%) GG: 21 (11.6%) GA: 59 (32.6%)	AA: 87 (50.0%) GG: 13 (7.5%) GA: 74 (42.5%)	355	p = .274
rs11924422	A: 209 (57.7%) C: 153 (42.3%)	A: 195 (56.0%) C: 153 (44.0%)	710	p = .647	AA: 60 (33.1%) CC: 32 (17.7%) CA: 89 (49.2%)	AA: 57 (32.8%) CC: 36 (20.7%) CA: 81 (46.6%)	355	p = .759
rs13083813	T: 221 (61.4%) A: 139 (38.6%)	T: 220 (63.2%) A: 128 (36.8%)	708	p = .617	TT: 68 (37.8%) AA: 27 (15.0%) AT: 85 (47.2%)	TT: 70 (40.2%) AA: 24 (13.8%) AT: 80 (46.0%)	354	p = .880
rs13075948*	C: 261 (72.1%) T: 101 (27.9%)	C: 254 (73.0%) T: 94 (27.0%)	710	p = .791	CC: 97 (53.6%) TT: 17 (9.4%) CT: 67 (37.0%)	CC: 90 (51.7%) TT: 10 (5.7%) CT: 74 (42.5%)	355	p = .725

Gene SNP	Allele Counts (%) Cases	Allele Counts (%) Controls	Total Alleles (*n*)	Allele Test[a]	Genotype Counts (%) Cases	Genotype Counts (%) Controls	Total Genotypes (*n*)	Genotype Test[b]
rs1155708	G: 247 (68.2%) A: 115 (31.8%)	G: 233 (67.3%) A: 113 (32.7%)	708	p = .806	GG: 87 (48.1%) AA: 21 (11.6%) GA: 73 (40.3%)	GG: 79 (45.7%) AA: 19 (11.0%) GA: 75 (43.4%)	354	p = .847
rs13086588	T: 246 (68.0%) G: 116 (32.0%)	T: 241 (69.3%) G: 107 (30.7%)	710	p = .708	TT: 87 (48.1%) GG: 22 (12.2%) GT: 72 (39.8%)	TT: 86 (49.4%) GG: 19 (10.9%) GT: 69 (39.7%)	355	p = .927
rs2082224*	G: 278 (76.8%) A: 84 (23.2%)	G: 265 (76.1%) A: 83 (23.9%)	710	p = .841	GG: 108 (59.7%) AA: 11 (6.1%) GA: 62 (34.3%)	GG: 100 (57.5%) AA: 9 (5.2%) GA: 65 (37.4%)	355	p = .674
rs1078985*	T: 271 (75.7%) C: 87 (24.3%)	T: 248 (72.9%) C: 92 (27.1%)	698	p = .403	TT: 106 (59.2%) CC: 14 (7.8%) TC: 59 (33.0%)	TT: 88 (51.8%) CC: 10 (5.9%) TC: 72 (42.4%)	349	p = .161
rs1036097	G: 188 (51.9%) A: 174 (48.1%)	G: 187 (53.7%) A: 161 (46.3%)	710	p = .632	GG: 49 (27.1%) AA: 42 (23.3%) GA: 90 (49.7%)	GG: 52 (29.9%) AA: 39 (22.4%) GA: 83 (47.7%)	355	p = .841
rs995435*	C: 263 (73.5%) T: 95 (26.5%)	C: 250 (74.0%) T: 88 (26.0%)	696	p = .888	CC: 99 (55.3%) TT: 15 (8.4%) CT: 65 (36.3%)	CC: 91 (53.8%) TT: 10 (5.9%) CT: 68 (40.2%)	348	p = .784
rs6792117	A: 185 (51.7%) G: 173 (48.3%)	A: 181 (52.0%) G: 167 (48.0%)	706	p = .920	AA: 48 (26.8%) GG: 42 (23.5%) GA: 89 (49.7%)	AA: 45 (25.9%) GG: 38 (21.8%) GA: 91 (52.3%)	353	p = .883
rs749794*	T: 252 (70.0%) C: 108 (30.0%)	T: 225 (66.2%) C: 115 (33.8%)	700	p = .277	TT: 90 (50.0%) CC: 18 (10.0%) TC: 72 (40.0%)	TT: 70 (41.2%) CC: 15 (8.8%) TC: 85 (50.0%)	350	p = .098
rs3773640*	A: 276 (76.2%) T: 86 (23.8%)	A: 262 (75.3%) T: 86 (24.7%)	710	p = .764	AA: 109 (60.2%) TT: 14 (7.7%) AT: 58 (32.0%)	AA: 95 (54.6%) TT: 7 (4.0%) AT: 72 (41.4%)	355	p = .284
rs3773644	C: 220 (60.8%) T: 142 (39.2%)	C: 220 (63.2%) T: 128 (36.8%)	710	p = .502	CC: 64 (35.4%) TT: 25 (13.8%) CT: 92 (50.8%)	CC: 69 (39.7%) TT: 23 (13.2%) CT: 82 (47.1%)	355	p = .702

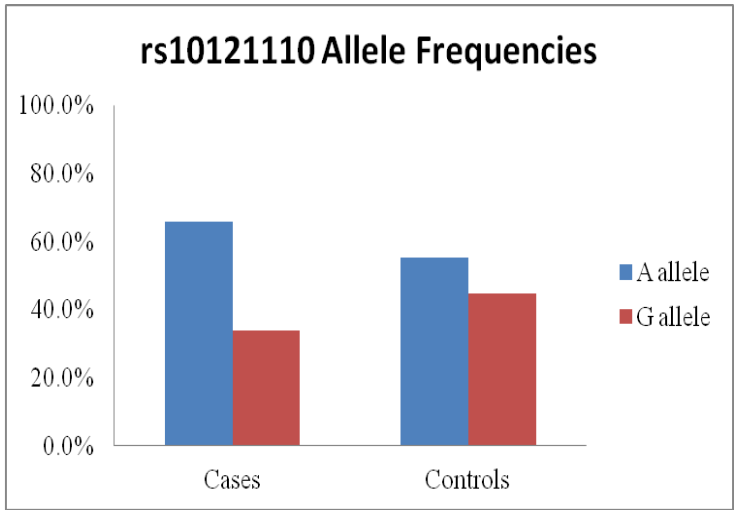
Gene SNP	Allele Counts (%) Cases	Allele Counts (%) Controls	Total Alleles (*n*)	Allele Test[a]	Genotype Counts (%) Cases	Genotype Counts (%) Controls	Total Genotypes (*n*)	Genotype Test[b]
rs3773645*	C: 253 (69.9%) G: 109 (30.1%)	C: 242 (69.9%) G: 104 (30.1%)	708	p > .999	CC: 87 (48.1%) GG: 15 (8.3%) CG: 79 (43.6%)	CC: 82 (47.4%) GG: 13 (7.5%) CG: 78 (45.1%)	354	p = .900
rs3773652	A: 196 (54.1%) G: 166 (45.9%)	A: 173 (49.7%) G: 175 (50.3%)	710	p = .237	AA: 57 (31.5%) GG: 42 (23.2%) AG: 82 (45.3%)	AA: 49 (28.2%) GG: 50 (28.7%) AG: 75 (43.1%)	355	p = .479
rs2043136*	T: 274 (75.7%) C: 88 (24.3%)	T: 246 (70.7%) C: 102 (29.3%)	710	p = .133	TT: 106 (58.6%) CC: 13 (7.2%) TC: 62 (34.3%)	TT: 87 (50.0%) CC: 15 (8.6%) TC: 72 (41.4%)	355	p = .105
rs1346907	C: 194 (53.6%) T: 168 (46.4%)	C: 180 (51.7%) T: 168 (48.3%)	710	p = .617	CC: 62 (34.3%) TT: 49 (27.1%) CT: 70 (38.7%)	CC: 52 (29.9%) TT: 46 (26.4%) CT: 76 (43.7%)	355	p = .582
rs876688	G: 239 (66.4%) A: 121 (33.6%)	G: 228 (65.5%) A: 120 (34.5%)	708	p = .806	GG: 85 (47.2%) AA: 26 (14.4%) GA: 69 (38.3%)	GG: 74 (42.5%) AA: 20 (11.5%) GA: 80 (46.0%)	354	p = .324
rs877572	G: 193 (53.3%) C: 169 (46.7%)	G: 186 (53.4%) C: 162 (46.6%)	710	p > .009	GG: 59 (32.6%) CC: 47 (26.0%) CG: 75 (41.4%)	GG: 53 (30.5%) CC: 41 (23.6%) CG: 80 (46.0%)	355	p = .686
rs9843942	G: 218 (60.6%) A: 142 (39.4%)	G: 221 (63.5%) A: 127 (36.5%)	708	p = .420	GG: 68 (37.8%) AA: 30 (16.7%) GA: 82 (45.6%)	GG: 69 (39.7%) AA: 22 (12.6%) GA: 83 (47.7%)	354	p = .565
rs3773663	G: 219 (60.5%) A: 143 (39.5%)	G: 210 (60.3%) A: 138 (39.7%)	710	p > .999	GG: 69 (38.1%) AA: 31 (17.1%) AG: 81 (44.8%)	GG: 62 (35.6%) AA: 26 (14.9%) AG: 86 (49.4%)	355	p = .662
rs744751*	C: 269 (74.3%) T: 93 (25.7%)	C: 253 (72.7%) T: 95 (27.3%)	710	p = .624	CC: 105 (58.0%) TT: 17 (9.4%) TC: 59 (32.6%)	CC: 90 (51.7%) TT: 11 (6.3%) TC: 73 (42.0%)	355	p = .234

Note. [a]$\chi 2$ test of independence testing association between allele and preeclampsia status; [b]$\chi 2$ test of independence testing association between SNP genotype (homozygote wildtype, homozygote variant, heterozygote) and preeclampsia status; *SNP genotypes dichotomized (homozygote wildtype, homozygote variant + heterozygote) due to small homozygote variant frequencies in either cases, controls, or both

a.



b.



c.

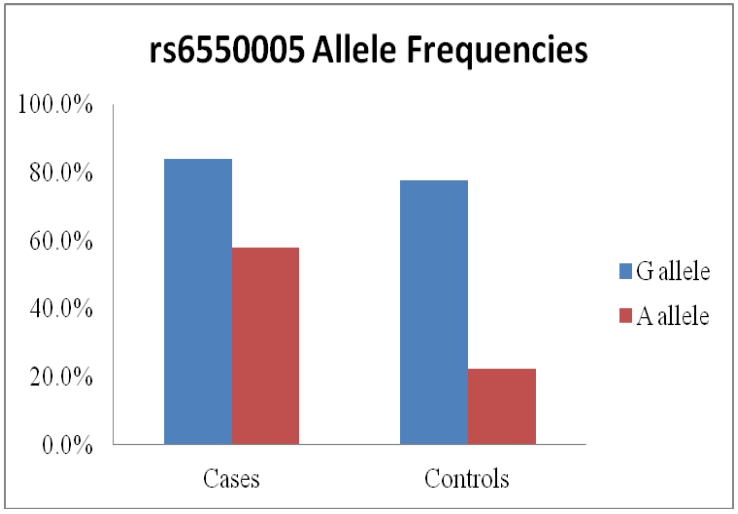


Figure 3. Frequency Distributions of tSNPs with Significant Allele Tests in the White Subgroup

3.4.1.4 Genotype test and logistic regression

Genotype test results are also presented in Table 9. In *ENG*, rs10121110 genotype was significantly associated with the development of preeclampsia (p = .022). This association was further explored with binary logistic regression and was evaluated with a more stringent criterion (α = 0.01; 99% CI) to account for multiple testing. Further analysis (Table 10) revealed that women homozygous for the A allele were 2.290 times more likely to develop preeclampsia compared to women homozygous for the G allele (β = .829, $\chi 2(1) = 6.993$, p = .008, exp(B) = 2.290, 99% CI [1.022, 5.133]). There was no significant difference in likelihood of developing preeclampsia between heterozygous women (AG) and women homozygous for the G allele (β = .419, $\chi 2(1) = 1.853$, p = .173, exp(B) = 1.521 , 99% CI [.688, 3.362]). In *TGFβR2*, rs6550005 genotype (dichotomized) was significantly associated with the development of preeclampsia (p = .039). Further exploration of this association with the stringent p-value criterion (α = 0.01; 99% CI) found no significant difference in the likelihood of developing preeclampsia between women homozygous for the G allele and the combined group of women that were homozygous for the A allele or heterozygous (β = .468, $\chi 2(1) = 4.239$, p = .039, exp(B) = 1.596, 99% CI [.889, 2.865]). The remaining genotype tests demonstrated no significant differences, but it is important to note that the genotype test for rs11792480 trended toward significance (p = .062).

Table 10. Logistic Regression Results for tSNPs with Significant Genotype Tests in White Subgroup

Gene/tSNP	Genotype Groups	OR	99% CI	p-value
ENG				
rs10121110	AA vs. GG	2.290	1.022 - 5.133	**.008**
	AG vs. GG	1.521	.688 - 3.362	.173
TGFBR2				
rs6550005*	GG vs. AA + GA	1.596	.889 - 2.865	.039

Note. tSNP = tagging SNP; OR = odds ratio; CI = confidence interval; * = SNP genotypes dichotomized (homozygote wildtype, homozygote variant + heterozygote) due to small homozygote variant frequencies in either cases, controls, or both

3.4.1.5 Linkage disequilibrium estimates & haplotype analysis

Pairwise comparison of linkage disequilibrium across the five tSNPs in *ENG* revealed no significant correlations with each other. R^2 values ranged from .007 (rs10121110 & rs10987746) to .637 (rs10760505 & rs10987746). The R^2 value for rs10121110 and rs11792480, which were both significantly associated with preeclampsia (rs10121110- allele and genotype test; rs11792480- allele test) and are separated by about 4000 bases, was .292. This indicates that these two SNPs are not in linkage disequilibrium with each other. Table 11 shows a correlation matrix of the R^2 values obtained from the pairwise comparisons.

Table 11: Correlation Matrix of *ENG* Pairwise Comparisons (R^2)

	rs10987746	rs10819309	rs10760505	rs11792480	rs10121110
rs10987746					
rs10819309	.562				
rs10760505	.637	.356			
rs11792480	.01	.011	.019		
rs10121110	.007	.008	.091	.292	

PLINK software estimated 19 possible haplotypes across the five *ENG* tSNPs. Only 17 of the 19 estimated haplotypes were present in the white sample. Table 12 displays the haplotype alleles and the frequencies of each allele in cases and controls. The order of the 5 tSNPs used in haplotype construction was as follows: rs10987746, rs10819309, rs10760505, rs11792480, rs10121110. We found that the haplotype distributions (CGTGA, TACAG, TACGA, and combined) were significantly different in cases and controls ($\chi 2(3) = 8.26$, $p = .041$). Further analysis revealed that TACGA, the haplotype containing the risks alleles from our significantly associated tSNPS, was over-represented in cases ($\chi 2(1) = 5.23$, $p = .022$) when comparing TACGA to all of the other alleles combined. The combined haplotype category (CGCAA, CGCAG, CGCGG, CGTAA, CGTAG, CGTGG, TACGG, TGCAG, TGCGA, CGCGA, TACAA, TGCGG, CATAA, TGTGG) was over-represented in controls ($\chi 2(1) = 5.75$, $p = .016$) when compared to the other alleles (CGTGA, TACAG, TACGA) combined. Pairwise comparisons of either CGTGA or TACAG compared to all of the other alleles combined were non-significant (data not shown). We did not find any significant differences in *ENG* diplotypes among cases and controls ($\chi 2(4) = 7.275$, $p = .122$). Table 13 demonstrates that there are differences in observed *ENG* haplotype frequencies among the white subgroup and black subgroup, with a 6.1% difference in TACGA frequency between the two groups (19.7% in white subgroup vs. 13.6% in black subgroup.

Table 12. *ENG* Haplotype Allele Frequencies in White Cases & Controls

Haplotype	Cases (*n* = 180 subjects) Allele Counts N (%)	Controls (*n* = 170 subjects) Allele Counts N (%)
CGTGA	94 (26.1%)	79 (23.2%)
TACAG	51 (14.2%)	51 (15.0%)
TACGA	83 (23.1%)	55 (16.2%)
Combined: CGCAA, CGCAG, CGCGG, CGTAA, CGTAG, CGTGG, TACGG, TGCAG, TGCGA, CGCGA, TACAA, TGCGG, CATAA, TGTGG	132 (36.7%)	155 (45.6%)

Note. tSNP order for haplotype assignment was rs10987746, rs10819309, rs10760505, rs11792480, rs10121110. Allele frequencies based on analysis of the most probable haplotypes for each subject. Haplotypes CATAG and TATAG (not listed above) did not represent the most probable haplotype for any of the subjects and were not included in any analyses.

Table 13. Comparison of Observed *ENG* Haplotype Frequencies Between Black & White Subgroups

	Haplotype	Black Total Subroup (*n* = 59 subjects) Allele Frequencies (%)	White Total Subgroup (*n* = 350 subjects) Allele Frequencies (%)
Both Subgroups	CGTGA	9.3%	24.7%
	TACAG	10.2%	14.6%
	TACGA	13.6%	19.7%
	CGCGG	23.7%	5.4%
	TACGG	8.5%	2.1%
	TGCAG	0.8%	4.4%
	TGCGA	4.2%	9.3%
	CGCGA	10.2%	0.4%
	TGCGG	16.1%	0.14%
Whites	CGCAA	0.0%	0.6%
	CGCAG	0.0%	4.4%
	CGTAA	0.0%	5.6%
	CGTAG	0.0%	2.6%
	CGTGG	0.0%	5.4%
	TACAA	0.0%	0.3%
	CATAA	0.0%	0.14%
	TGTGG	0.0%	0.14%
Blacks	CGTGG	2.5%	0.0%
	TGCAA	0.8%	0.0%

Note. The haplotype frequencies were based on what was observed in the subjects using the most probable haplotypes for each subject. One subject from the black subgroup and five subjects from the white subgroup did not have available haplotypes.

3.4.1.6 Clinical characteristics by SNP genotype assignment

Blood pressure measurements and pre-pregnancy BMI were significantly different in cases and controls (Table 7). Given these results, we further examined whether genotype assignment of the tSNPs with significant genotype tests (rs10121110 and rs6550005) were associated with these clinical characteristics. Neither average SBP less than 20 weeks' gestation ($F(2, 328) = .500$, $p = .607$), average DBP less than 20 weeks' ($F(2, 177.984) = .265$, $p = .767$), average SBP in labor

(F(2, 172.976) = 2.137, p = .121), average DBP in labor (F(2, 344) = 1.767, p = .172), or pre-pregnancy BMI (H(2) = 1.321, p = .517) were significantly different between rs10121110 genotype groups. Moreover, rs6550005 genotype assignment (dichotomized) was not significantly associated with average SBP less than 20 weeks' gestation (t(336) = -1.196, p = .233), average DBP less than 20 weeks' (t(336) = -.563, p = .574), average SBP in labor (U = 12198.0, p = .066), average DBP in labor (t(352) = 1.552, p = .122), or pre-pregnancy BMI (U = 13565.5, p = .840).

3.4.2 Black subgroup

3.4.2.1 Demographic and clinical characteristics

Demographic and clinical characteristics for the black subgroup comprised of 30 cases and 30 controls (Figure 4) are presented in Table 14. Cases and controls did not significantly differ for variables on which they were matched (age, parity). Unlike what we observed in the white subgroup, women with preeclampsia were not significantly different from healthy controls with respect to pre-pregnancy BMI, average systolic and diastolic blood pressure at < 20 weeks' gestation, or percent of cesarean section deliveries in the black subgroup. Women with preeclampsia had a higher average blood pressure in labor (SBP: M = 159.9 mmHg vs. M = 120.7 mmHg, p < .001; DBP: M = 97.1 mmHg vs. M = 72.6 mmHg, p < .001) and delivered at an earlier gestational age (M = 36.9 weeks vs. M = 40.6 weeks, p < .001) compared to healthy controls. Babies born to women with preeclampsia were of lower birthweight (M = 2313.9 grams vs. M = 3388.8 grams, p <.001) and were more likely to be small for gestational age (33.3% vs. 3.3%, p = .003).

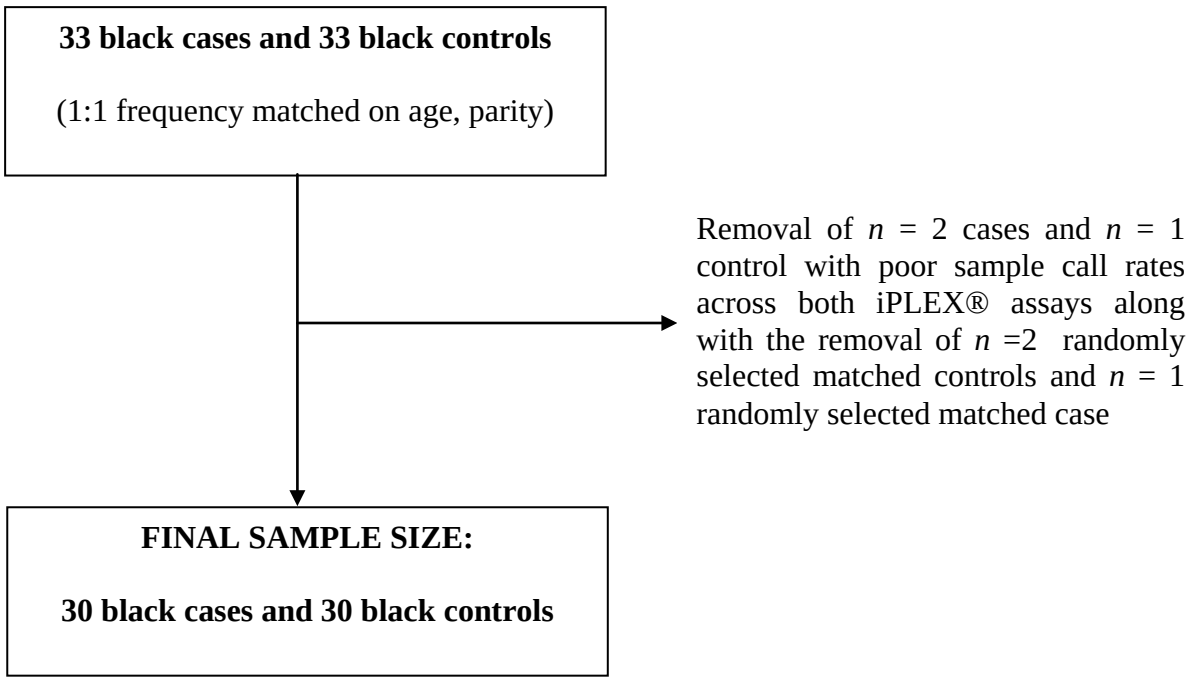


Figure 4. Sample Flow Chart of Black Subgroup

Table 14. Demographic and Clinical Characteristics of Black Subgroup

Variable	Cases (*n* = 30)	Controls (*n* = 30)	p-value
Maternal age (*Mdn (min-max)*, years)	20.0 (14-37)	20.0 (14-37)	.988[a]
Gravida (*Mdn (min-max)*)	1 (1-6)	1 (1-6)	.384[a]
Nulliparous (*n, %)*	25 (83.3%)	25 (83.3%)	-------
Gestational age at delivery (*Mdn (min-max)*, weeks)	36.9 (27.4-40.0)	40.6 (37.1-42.1)	**< .001**[a]
Birthweight (*M (SD)*, grams)	2313.9 (715.8)	3388.8 (405.3)	**< .001**[b]
Caesarean section (*n, %*)[c]	17 (65.4%)	19 (70.4%)	.697[d]
Small for gestational age (*n, %*)	10 (33.3%)	1 (3.3%)	**.003**[d]
Avg. SBP < 20 weeks gestation (*M (SD)*, mmHg)[e]	113.3 (9.2)	114.4 (6.8)	.599[f]
Avg. DBP < 20 weeks gestation (*M (SD)*, mmHg)[e]	70.4 (6.5)	69.2 (4.2)	.405[f]
Avg. SBP in labor (*M (SD)*, mmHg)[g]	159.9 (17.8)	120.7 (9.1)	**< .001**[b]
Avg. DBP in labor (*M (SD)*, mmHg)[g]	97.1 (10.4)	72.6 (7.4)	**< .001**[f]
Pre-pregnancy BMI (*Mdn (min-max)*)[h]	23.0 (17.7-38.4)	25.8 (19.4-49.9)	.250[a]

Note. [a]Mann-Whitney U test; [b]Independent samples t-test with unequal variances; [c]*n* = 26 cases and *n* = 27 controls due to missing data; [d]$\chi 2$ test of independence; SBP = systolic blood pressure; mmHg = millimeters of mercury; [e]*n* = 24 cases and *n* = 29 controls due to missing data; [f]Independent samples t-test; DBP = diastolic blood pressure; [g]*n* = 30 cases and *n* = 29 controls due to missing data; [h]*n* = 27 cases and *n* = 30 controls due to missing data

3.4.2.2 Genotype call rates, MAF, HWE

Descriptive information for each tSNP and functional SNP is provided in Table 15. tSNPS rs8179181 and rs3087465 could not be genotyped and functional SNP rs11477314 was essentially monomorphic in the black subgroup, resulting in their omission from analysis. The remaining 46 SNPs had genotype call rates that ranged from 98.3% - 100%. Of the 46 SNPs analyzed, 3 tSNPs violated HWE ($p < .05$) in the black subgroup. One tSNP was located in

ALK1 (rs11169953) and two tSNPs were located in *TGFβR2* (rs12487185 and rs1078985). Separate evaluation of HWE in cases and controls revealed that rs11169953 was in HWE in cases and controls separately (p = .119 & p = .145), rs12487185 was in HWE in cases (p = .099), and rs1078985 was in HWE in cases and controls separately (p = .155 & p = .165).

Table 15. tSNP and Functional SNP Information in Black Subgroup (*N* = 60)

Gene-Chromosome tSNP	Wildtype Allele/ Nucleotide #/ Variant Allele[a]	*n for each SNP*	Study MAF	HapMap MAF	HWE[b]
ALK5(TGFBR1)-Chr9					
rs6478974	T/101874403/A	59	A = .237	A = .075	p = .276
rs10739778	A/101875789/C	59	C = .322	C = .339	p = .597
rs420549	G/101914873/C	59	C = .119	C = .106	p = .582
ALK1-Chr12					
rs3759178	T/52299259/G	59	G = .398	T = .491	p = .146
rs11169953	C/52304399/T	59	T = .466	T = .482	**p = .028**
rs706819	G/52315923/A	59	A = .297	A = .236	p = .899
TGFB1-Chr19					
rs8179181	--/41838206/--	----	----	----	----
rs4803455	C/41851509/A	59	A = .492	C = .397	p = .157
rs11466314	G/41860236/A	59	A = .051	A = .025	----
rs1800469	C/41860296/T	59	T = .271	T = .208	p = .741
rs1800468	C/41860587/T	60	T = .050	T = .033	p > .999
rs4803457	C/41861359/T	59	T = .483	T = .420	p = .237
ENG-Chr9					
rs10987746	T/130580093/C	59	C = .458	C = .438	p = .371
rs10819309	G/130581723/A	59	A = .322	A = .195	p = .249
rs10760505	C/130589853/T	59	T = .119	T = .058	p = .169
rs11792480	G/130598125/A	59	A = .119	A = .004	p = .169
rs10121110	G/130602408/A	59	A = .381	A = .310	p = .823
TGFBR2-Chr3					
rs3087465	--/30647160/--	----	----	----	----
rs6550005	G/30650064/A	60	A = .367	G = .491	p = .294
rs11129420	A/30658541/T	60	T = .292	T = .097	p = .572
rs6802220	A/30659652/G	60	G = .358	G = .243	p = .484
rs17025785	T/30667425/C	60	C = .300	C = .283	p = .380
rs4522809	T/30668684/C	60	C = .475	C = .411	p = .069
rs4955212	C/30669358/T	60	T = .208	T = .208	p = .429
rs5020833	C/30670425/G	60	G = .292	G = .142	p = .233
rs6809777	C/30672362/T	60	T = .225	T = .204	p = .147

Gene-Chromosome tSNP	Wildtype Allele/ Nucleotide #/ Variant Allele[a]	*n for each SNP*	Study MAF	HapMap MAF	HWE[b]
rs12487185	A/30677269/G	60	G = .200	G = .088	**p = .007**
rs11924422	A/30677484/C	60	C = .367	C = .381	p = .554
rs13083813	T/30679558/A	60	A = .225	A = .075	p = .261
rs13075948	C/30683506/T	60	T = .192	T = .155	p > .999
rs1155708	G/30686740/A	60	A = .392	A = .358	p = .913
rs13086588	T/30688757/G	60	G = .375	G = .319	p = .403
rs2082224	G/30689755/A	60	A = .267	A = .243	p > .999
rs1078985	T/30690911/C	59	C = .169	C = .221	**p = .047**
rs1036097	G/30693643/A	60	A = .333	A = .292	p = .856
rs995435	C/30700922/T	59	T = .356	T = .429	p = .390
rs6792117	A/30704007/G	60	G = .383	G = .363	p = .655
rs749794	T/30708432/C	59	C = .381	C = .451	p = .442
rs3773640	A/30709511/T	60	T = .250	T = .248	p = .487
rs3773644	C/30712344/T	60	T = .325	T = .332	p = .856
rs3773645	C/30712460/G	60	G = .225	G = .111	p = .055
rs3773652	G/30718942/A	60	A = .467	A = .292	p = .277
rs2043136	T/30720304/C	60	C = .225	C = .279	p = .712
rs1346907	C/30723470/T	60	T = .283	T = .221	p > .001
rs876688	G/30725776/A	60	A = .433	A = .429	p = .517
rs877572	G/30726432/C	60	C = .292	C = .246	p = .549
rs9843942	G/30729636/A	60	A = .467	G = .385	p = .310
rs3773663	G/30730872/A	60	A = .450	A = .385	p = .938
rs744751	C/30735937/T	60	T = .125	T = .058	p > .001

Note. MAF = mean allele frequency; HWE= hardy weinberg equilibrium; [a]wildtype and variant alleles based on study sample, nucleotide # obtained from UCSC Genome Browser assembly Feb. 2009 (GRCh37/hg19) (Fujita et al., 2011); [b]$\chi 2$ goodness-of-fit test or exact test (significant results bolded); ---- = not analyzed

3.4.2.3 Allele test

Allele test results are presented in Table 16. In *TGFβR1*, the allelic distribution for rs10739778 was significantly different in cases and controls (Figure 5a). Compared to controls, the A allele was over-represented in cases (79.3% vs. 56.7%, p = .008). In *TGFβR2*, allelic frequency distributions for rs6550005, rs1346907, and rs877572 were significantly different in cases and controls (Figure 5b-d). Compared to controls, the A allele of rs6550005 was over-represented in cases (46.7% vs. 26.7%, p = .023), the C allele of rs1346907 was over-represented in cases

(70.0% vs. 63.3%, p = .043), and the G allele of rs877572 was over-represented in cases (70.0% vs. 61.7%, p = .027). Allele tests for the remaining tSNPs and functional SNP were non-significant.

Table 16. Results of Association Analysis in Black Subgroup (Total N = 60)

Gene SNP	Allele Counts (%) Cases	Allele Counts (%) Controls	Total Alleles (*n*)	Allele Test[a]	Genotype Counts (%) Cases	Genotype Counts (%) Controls	Total Genotypes (*n*)	Genotype Test[b]
ALK5 (TGFBR1)								
rs6478974*	T: 40 (69.0%) A: 18 (31.0%)	T: 50 (83.3%) A: 10 (16.7%)	118	p = .067	TT: 15 (51.7%) AA: 4 (13.8%) TA: 10 (34.5%)	TT: 21 (70.0%) AA: 1 (3.3%) TA: 8 (26.7%)	59	p = .150
rs10739778	A: 46 (79.3%) C: 12 (20.7%)	A: 34 (56.7%) C: 26 (43.3%)	118	**p = .008**	AA: 19 (65.5%) CC: 2 (6.9%) AC: 8 (27.6%)	AA: 9 (30.0%) CC: 5 (16.7%) AC: 16 (53.3%)	59	**p = .028**
rs420549*	G: 53 (91.4%) C: 5 (8.6%)	G: 51 (85.0%) C: 9 (15.0%)	118	p = .284	GG: 24 (82.8%) CC: 0 (0.0%) GC: 5 (17.2%)	GG: 22 (73.3%) CC: 1 (3.3%) GC: 7 (23.3%)	59	p = .383
ALK1								
rs3759178	T: 33 (56.9%) G: 25 (43.1%)	T: 38 (63.3%) G: 22 (36.7%)	118	p = .475	TT: 10 (34.5%) GG: 6 (20.7%) GT: 13 (44.8%)	TT: 14 (46.7%) GG: 6 (20.0%) GT: 10 (33.3%)	59	p = .594
rs11169953	C: 34 (58.6%) T: 24 (41.4%)	C: 29 (48.3%) T: 31 (51.7%)	118	p = .264	CC: 12 (41.4%) TT: 7 (24.1%) CT: 10 (34.5%)	CC: 9 (30.0%) TT: 10 (33.3%) CT: 11 (36.7%)	59	p = .610
rs706819*	G: 43 (74.1%) A: 15 (25.9%)	G: 40 (66.7%) A: 20 (33.3%)	118	p = .374	GG: 16 (55.2%) AA: 2 (6.9%) GA: 11 (37.9%)	GG: 13 (43.3%) AA: 3 (10.0%) GA: 14 (46.7%)	59	p = .363
TGFB1								
rs4803455	C: 33 (56.9%) A: 25 (43.1%)	C: 27 (45.0%) A: 33 (55.0%)	118	p = .196	CC: 8 (27.6%) AA: 4 (13.8%) CA: 17 (58.6%)	CC: 10 (33.3%) AA: 13 (43.3%) CA: 7 (23.3%)	59	**p = .010**
rs1800469*	C: 40 (69.0%) T: 18 (31.0%)	C: 46 (76.7%) T: 14 (23.3%)	118	p = .348	CC: 13 (44.8%) TT: 2 (6.9%) CT: 14 (48.3%)	CC: 19 (63.3%) TT: 3 (10.0%) CT: 8 (26.7%)	59	p = .154

Gene SNP	Allele Counts (%) Cases	Allele Counts (%) Controls	Total Alleles (*n*)	Allele Test[a]	Genotype Counts (%) Cases	Genotype Counts (%) Controls	Total Genotypes (*n*)	Genotype Test[b]
rs1800468*	C: 57 (95.0%) T: 3 (5.0%)	C: 57 (95.0%) T: 3 (5.0%)	120	p > .999	CC: 27 (90.0%) TT: 0 (0.0%) CT: 3 (10.0%)	CC: 27 (90.0%) TT: 0 (0.0%) CT: 3 (10.0%)	60	p > .999
rs4803457	C: 25 (43.1%) T: 33 (56.9%)	C: 36 (60.0%) T: 24 (40.0%)	118	p = .066	CC: 4 (13.8%) TT: 8 (27.6%) CT: 17 (58.6%)	CC: 14 (46.7%) TT: 8 (26.7%) CT: 8 (26.7%)	59	**p = .012**
ENG								
rs10987746	T: 31 (53.4%) C: 27 (46.6%)	T: 33 (55.0%) C: 27 (45.0%)	118	p = .862	TT: 9 (31.0%) CC: 7 (24.1%) TC: 13 (44.8%)	TT: 10 (33.3%) CC: 7 (23.3%) TC: 13 (43.3)	59	p = .982
rs10819309	G: 41 (70.7%) A: 17 (29.3%)	G: 39 (65.0%) A: 21 (35%)	118	p = .507	GG: 16 (55.2%) AA: 4 (13.8%) GA: 9 (31.0%)	GG: 13 (43.3%) AA: 4 (13.3%) GA: 13 (43.3%)	59	p = .590
rs10760505*	C: 50 (86.2%) T: 8 (13.8%)	C: 54 (90.0%) T: 6 (10.0%)	118	p = .522	CC: 22 (75.9%) TT: 1 (3.4%) CT: 6 (20.7%)	CC: 25 (83.3%) TT: 1 (3.3%) CT: 4 (13.3%)	59	p = .476
rs11792480*	G: 49 (84.5%) A: 9 (15.5%)	G: 55 (91.7%) A: 5 (8.3%)	118	p = .227	GG: 22 (75.9%) AA: 2 (6.9%) AG: 5 (17.2%)	GG: 25 (83.3%) AA: 0 (0.0%) AG: 5 (16.7%)	59	p = .476
rs10121110	G: 37 (63.8%) A: 21 (36.2%)	G: 36 (60.0%) A: 24 (40.0%)	118	p = .671	GG: 13 (44.8%) AA: 5 (17.2%) AG: 11 (37.9%)	GG: 10 (33.3%) AA: 4 (13.3%) AG: 16 (53.3%)	59	p = .472
TGFBR2								
rs6550005	G: 32 (53.3%) A: 28 (46.7%)	G: 44 (73.3%) A: 16 (26.7%)	120	**p = .023**	GG: 9 (30.0%) AA: 7 (23.3%) GA: 14 (46.7%)	GG: 17 (56.7%) AA: 3 (10.0%) GA: 10 (33.3%)	60	p = .094
rs11129420	A: 40 (66.7%) T: 20 (33.3%)	A: 45 (75.0%) T: 15 (25.0%)	120	p = .315	AA: 13 (43.3%) TT: 3 (10.0%) TA: 14 (46.7%)	AA: 18 (60.0%) TT: 3 (10.0%) TA: 9 (30.0%)	60	p = .419

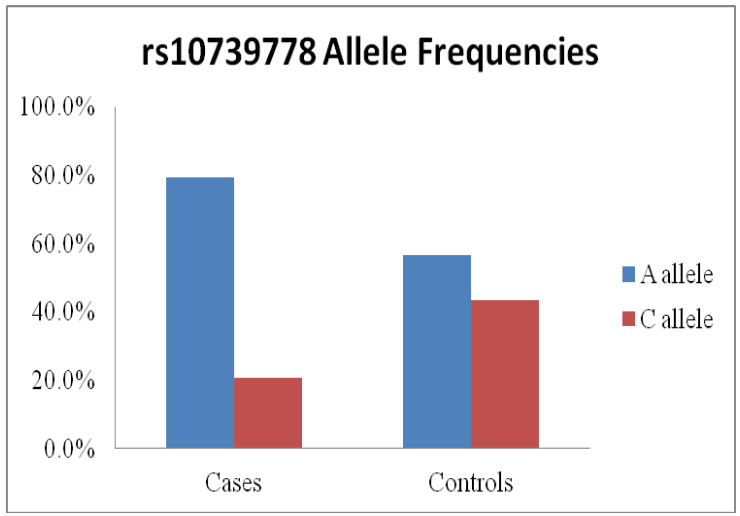
Gene SNP	Allele Counts (%) Cases	Allele Counts (%) Controls	Total Alleles (*n*)	Allele Test[a]	Genotype Counts (%) Cases	Genotype Counts (%) Controls	Total Genotypes (*n*)	Genotype Test[b]
rs6802220	A: 37 (61.7%) G: 23 (38.3%)	A: 40 (66.7%) G: 20 (33.3%)	120	p = .566	AA: 11 (36.7%) GG: 4 (13.3%) AG: 15 (50.0%)	AA: 15 (50.0%) GG: 5 (16.7%) AG: 10 (33.3%)	60	p = .418
rs17025785*	T: 42 (70.0%) C: 18 (30.0%)	T: 42 (70.0%) C: 18 (30.0%)	120	p > .999	TT: 14 (46.7%) CC: 2 (6.7%) TC: 14 (46.7%)	TT: 14 (46.7%) CC: 2 (6.7%) TC: 14 (46.7%)	60	p > .999
rs4522809	T: 33 (55.0%) C: 27 (45.0%)	T: 30 (50.0%) C: 30 (50.0%)	120	p = .584	TT: 8 (26.7%) CC: 5 (16.7%) CT: 17 (56.7%)	TT: 5 (16.7%) CC: 5 (16.7%) CT: 20 (66.7%)	60	p = .626
rs4955212*	C: 48 (80.0%) T: 12 (20.0%)	C: 47 (78.3%) T: 13 (21.7%)	120	p = .823	CC: 18 (60.0%) TT: 0 (0%) CT: 12 (40.0%)	CC: 18 (60.0%) TT: 1 (3.3%) CT: 11 (36.7%)	60	p > .999
rs5020833*	C: 45 (75.0%) G: 15 (25.0%)	C: 40 (66.7%) G: 20 (33.3%)	120	p = .315	GG: 17 (56.7%) AA: 2 (6.7%) GA: 11 (36.7%)	GG: 15 (50.0%) AA: 5 (16.7%) GA: 10 (33.3%)	60	p = .605
rs6809777*	C: 48 (80.0%) T: 12 (20.0%)	C: 45 (75.0%) T: 15 (25.0%)	120	p = .512	CC: 21 (70.0%) TT: 3 (10.0%) CT: 6 (20.0%)	CC: 17 (56.7%) TT: 2 (6.7%) CT: 11 (36.7%)	60	p = .422
rs12487185*	A: 51 (85.0%) G: 9 (15.0%)	A: 45 (75.0%) G: 15 (25.0%)	120	p = .170	AA: 23 (76.7%) GG: 2 (6.7%) GA: 5 (16.7%)	AA: 19 (63.3%) GG: 4 (13.3%) GA: 7 (23.3%)	60	p = .260
rs11924422*	A: 33 (55.0%) C: 27 (45.0%)	A: 43 (71.7%) C: 17 (28.3%)	120	p = .058	AA: 8 (26.7%) CC: 5 (16.7%) CA: 17 (56.7%)	AA: 15 (50.0%) CC: 2 (6.7%) CA: 13 (43.3%)	60	p = .063
rs13083813*	T: 44 (73.3%) A: 16 (26.7%)	T: 49 (81.7%) A: 11 (18.3%)	120	p = .275	TT: 15 (50.0%) AA: 1 (3.3%) AT: 14 (46.7%)	TT: 19 (63.3%) AA: 0 (0.0%) AT: 11 (36.7%)	60	p = .297
rs13075948*	C: 48 (70.0%) T: 12 (30.0%)	C: 49 (81.7%) T: 11 (18.3%)	120	p = .823	CC: 19 (63.3%) TT: 1 (3.3%) CT: 10 (33.3%)	CC: 20 (66.7%) TT: 1 (3.3%) CT: 9 (30.0%)	60	p = .787

Gene SNP	Allele Counts (%) Cases	Allele Counts (%) Controls	Total Alleles (*n*)	Allele Test[a]	Genotype Counts (%) Cases	Genotype Counts (%) Controls	Total Genotypes (*n*)	Genotype Test[b]
rs1155708	G: 39 (65.0%) A: 21 (35.0%)	G: 34 (56.7%) A: 26 (43.3%)	120	p = .351	GG: 12 (40.0%) AA: 3 (10.0%) GA: 15 (50.0%)	GG: 10 (33.3%) AA: 6 (20.0%) GA: 14 (46.7%)	60	p = .611
rs13086588	T: 41 (68.3%) G: 19 (31.7%)	T: 34 (56.7%) G: 26 (43.3%)	120	p = .187	TT: 14 (46.7%) GG: 3 (10.0%) GT: 13 (43.3%)	TT: 11 (36.7%) GG: 7 (23.3%) GT: 12 (40.0%)	60	p = .368
rs2082224*	G: 46 (76.7%) A: 14 (23.3%)	G: 42 (70.0%) A: 18 (30.0%)	120	p = .410	GG: 17 (56.7%) AA: 1 (3.3%) GA: 12 (40.0%)	GG: 15 (50.0%) AA: 3 (10.0%) GA: 12 (40.0%)	60	p = .605
rs1078985*	T: 50 (83.3%) C: 10 (16.7%)	T: 48 (82.8%) C: 10 (17.2%)	118	p = .920	TT: 22 (73.3%) CC: 2 (6.7%) TC: 6 (20.0%)	TT: 21 (72.4%) CC: 2 (6.9%) TC: 6 (20.7%)	59	p = .937
rs1036097*	G: 39 (65.0%) A: 21 (35.0%)	G: 41 (68.3%) A: 19 (31.7%)	120	p = .699	GG: 11 (36.7%) AA: 2 (6.7%) GA: 17 (56.7%)	GG: 16 (53.3%) AA: 5 (16.7%) GA: 9 (30.0%)	60	p = .194
rs995435	C: 40 (66.7%) T: 20 (33.3%)	C: 36 (62.1%) T: 22 (37.9%)	118	p = .603	CC: 14 (46.7%) TT: 4 (13.3%) CT: 12 (40.0%)	CC: 12 (41.4%) TT: 5 (17.2%) CT: 12 (41.4%)	59	p = .940
rs6792117	A: 35 (58.3%) G: 25 (41.7%)	A: 39 (65.0%) G: 21 (35.0%)	120	p = .454	AA: 8 (26.7%) GG: 3 (10.0%) GA: 19 (63.3%)	AA: 14 (46.7%) GG: 5 (16.7%) GA: 11 (36.7%)	60	p = .140
rs749794*	T: 41 (70.7%) C: 17 (29.3%)	T: 32 (53.3%) C: 28 (46.7%)	118	p = .052	TT: 14 (48.3%) CC: 2 (6.9%) TC: 13 (44.8%)	TT: 10 (33.3%) CC: 8 (26.7%) TC: 12 (40.0%)	59	p = .243
rs3773640*	A: 47 (78.3%) T: 13 (21.7%)	A: 43 (71.7%) T: 17 (28.3%)	120	p = .399	AA: 20 (66.7%) TT: 3 (10.0%) AT: 7 (23.3%)	AA: 15 (50.0%) TT: 2 (6.7%) AT: 13 (43.3%)	60	p = .190
rs3773644*	C: 37 (61.7%) T: 23 (38.3%)	C: 44 (73.3%) T: 16 (26.7%)	120	p=.173	CC: 11 (36.7%) TT: 4 (13.3%) CT: 15 (50.0%)	CC: 16 (53.3%) TT: 2 (6.7%) CT: 12 (40.0%)	60	p = .194

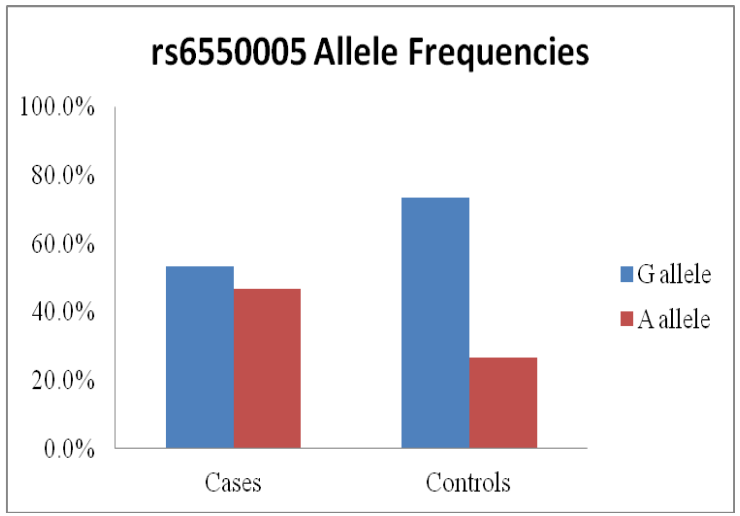
Gene SNP	Allele Counts (%) Cases	Allele Counts (%) Controls	Total Alleles (*n*)	Allele Test[a]	Genotype Counts (%) Cases	Genotype Counts (%) Controls	Total Genotypes (*n*)	Genotype Test[b]
rs3773645*	C: 45 (75.0%) G: 15 (25.0%)	C: 48 (70.0%) G: 12 (30.0%)	120	p = .512	CC: 20 (66.7%) GG: 5 (16.7%) CG: 5 (16.7%)	CC: 19 (63.3%) GG: 1 (3.3%) CG: 10 (33.3%)	60	p = .787
rs3773652	G: 30 (50.0%) A: 30 (50.0%)	G: 34 (56.7%) A: 26 (43.3%)	120	p = .462	GG: 7 (23.3%) AA: 7 (23.3%) AG: 16 (53.3%)	GG: 8 (26.7%) AA: 4 (13.3%) AG: 18 (60.0%)	60	p = .606
rs2043136*	T: 48 (70.0%) C: 12 (30.0%)	T: 45 (75.0%) C: 15 (15.0%)	120	p = .512	TT: 19 (63.3%) CC: 1 (3.3%) TC: 10 (33.3%)	TT: 16 (53.3%) CC: 1 (3.3%) TC: 13 (43.3%)	60	p = .432
rs1346907*	C: 48 (70.0%) T: 12 (30.0%)	C: 38 (63.3%) T: 22 (36.7%)	120	**p = .043**	CC: 18 (60.0%) TT: 0 (0%) CT: 12 (40.0%)	CC: 13 (43.3%) TT: 5 (16.7%) CT: 12 (40.0%)	60	p = .196
rs876688	G: 32 (53.3%) A: 28 (46.7%)	G: 36 (60.0%) A: 24 (40.0%)	120	p = .462	GG: 7 (23.3%) AA: 5 (16.7%) GA: 18 (60.0%)	GG: 11 (36.7%) AA: 5 (16.7%) GA: 14 (46.7%)	60	p = .499
rs877572*	G: 48 (70.0%) C: 12 (30.0%)	G: 37 (61.7%) C: 23 (38.3%)	120	**p = .027**	GG: 18 (60.0%) CC: 0 (0.0%) CG: 12 (40.0%)	GG: 13 (43.3%) CC: 6 (20.0%) CG: 11 (36.7%)	60	p = .196
rs9843942	G: 35 (58.3%) A: 25 (41.7%)	G: 29 (48.3%) A: 31 (51.7%)	120	p = .271	GG: 11 (36.7%) AA: 6 (20.0%) GA: 13 (43.3%)	GG: 8 (26.7%) AA: 9 (30.0%) GA: 13 (43.3%)	60	p = .585
rs3773663	G: 28 (46.7%) A: 32 (53.3%)	G: 38 (63.3%) A: 22 (36.7%)	120	p = .066	GG: 6 (20.0%) AA: 8 (26.7%) AG: 16 (53.3%)	GG: 12 (40.0%) AA: 4 (13.3%) AG: 14 (46.7%)	60	p = .177
rs744751*	C: 50 (83.3%) T: 10 (16.7%)	C: 55 (91.7%) T: 5 (8.3%)	120	p = .168	CC: 21 (70.0%) TT: 1 (3.3%) TC: 8 (26.7%)	CC: 25 (83.3%) TT: 0 (0.0%) TC: 5 (16.7%)	60	p = .222

Note. [a]$\chi 2$ test of independence or Fisher's exact test testing association between allele and preeclampsia status; [b]$\chi 2$ test of independence or Fisher's Exact exact test testing association between SNP genotype (homozygote wildtype, homozygote variant, heterozygote) and preeclampsia status; *SNP genotypes dichotomized (homozygote wildtype, homozygote variant + heterozygote) due to small homozygote variant frequencies in either cases, controls, or both

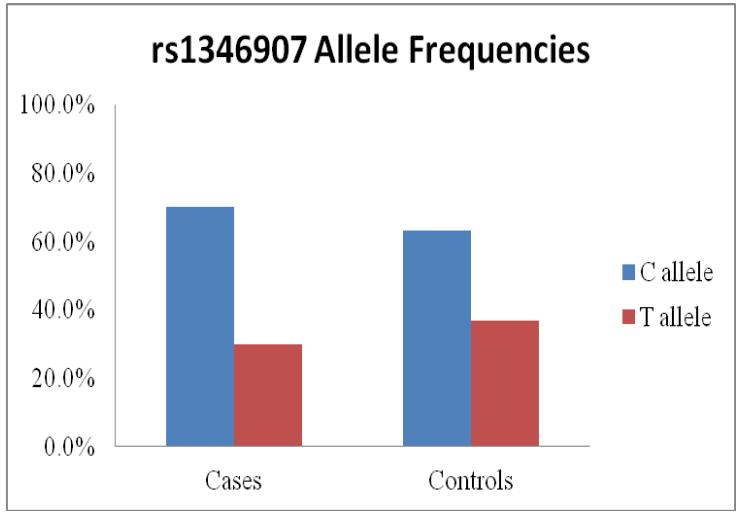
a.



b.



c.



d.

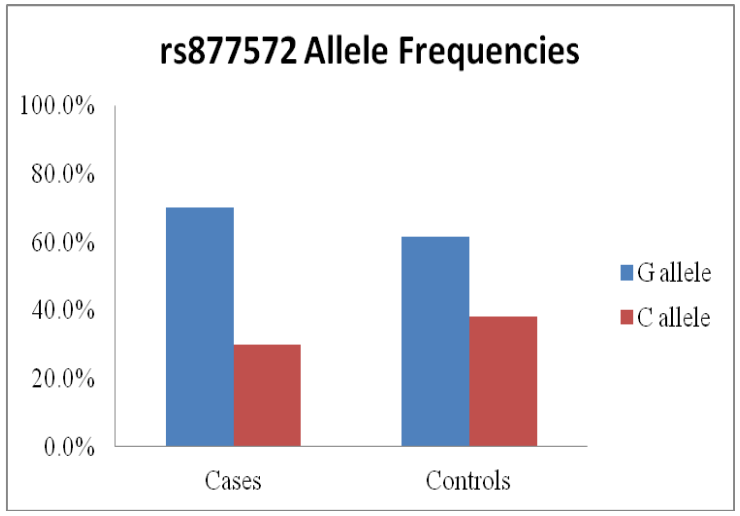


Figure 5. Frequency Distributions of tSNPs with Significant Allele Tests in the Black Subgroup

3.4.2.4 Genotype test and logistic regression

Genotype test results are also presented in Table 16. In *TGFβ1*, rs4803455 genotype was significantly associated with the development of preeclampsia (p = .010). This association was further explored with binary logistic regression and was evaluated with a more stringent criterion (α = .01; 99% CI) to account for multiple testing. Further analysis of rs4803455 (Table 17) revealed no significant difference in likelihood of developing preeclampsia between women homozygous for the A allele and women homozygous for the C allele (β = -.956, $\chi 2$(1) = 1.654, p = .198, exp(B) = .385 , 99% CI [.057, 2.607]). Moreover, there was no significant difference in likelihood of developing preeclampsia between heterozygous women (CA) and women homozygous for the C allele (β = 1.110, $\chi 2$(1) = 2.890, p = .089, exp(B) = 3.036 , 99% CI [.564, 16.330]). In addition to rs4803455, *TGFβ1* tSNP rs4803457 genotype was also significantly associated with the development of preeclampsia (p = .012). Further analysis of rs4803457 revealed that heterozygous women (CT) were 7.437 times more likely to develop preeclampsia compared to women homozygous for the C allele (β = 2.007, $\chi 2$(1) = 7.969, p = .005, exp(B) = 7.437, 99% CI [1.192, 46.408]). There was no significant difference in likelihood of developing preeclampsia between women homozygous for the T allele and women homozygous for the C allele (β = 1.253, $\chi 2$(1) = 2.746, p = .097, exp(B) = 3.500, 99% CI [.499, 24.530]). Lastly, in *TGFβR1*, rs10739778 genotype was significantly associated with the development of preeclampsia (p = .028). Further exploration of this association with the stringent p-value criterion (α = .01; 99% CI) found no significant difference in the likelihood of developing preeclampsia between heterozygous women (AC) and women homozygous for the A allele (β = -1.440, $\chi 2$(1) = 5.907, p = .015, exp(B) = .237, 99% CI [.051, 1.090]). Moreover, there was no significant difference in likelihood of developing preeclampsia between women homozygous for

the C allele and women homozygous for the A allele (β = -1.664, $\chi 2(1)$ = 3.204, p = .073, exp(B) = .189, 99% CI [.017, 2.076]). Genotype tests for the remaining tSNPs demonstrated no significant differences; however, the genotype test for rs11924422 trended toward significance (p = .063).

Table 17. Logistic Regression Results for tSNPS with Significant Genotype Tests in Black Subgroup

Gene/tSNP	Genotype Groups	OR	99% CI	p-value
TGFβ1				
rs4803455	AA vs. CC	.385	.057 - 2.607	p = .198
	CA vs. CC	3.036	.564 - 16.330	p = .089
rs4803457	TT vs. CC	3.500	.499 - 24.530	p = .097
	CT vs. CC	7.437	1.192 - 46.408	**p = .005**
TGFβR1				
rs10739778	CC vs. AA	.189	.017 - 2.076	p = .073
	AC vs. AA	.237	.051 - 1.090	p = .015

Note. tSNP = tagging SNP; OR = odds ratio; CI = confidence interval

3.4.2.5 Clinical characteristics by SNP genotype assignment

In the black subgroup, average blood pressure measurement during labor was significantly different between cases and controls, but pre-pregnancy BMI and average blood pressure measurements < 20 weeks' gestation were not statistically different (Table 14). Because a small sample size may lack the needed power to detect significant differences, we decided to include pre-pregnancy BMI and all blood pressure measurement variables in our examination of the association between tSNP genotype assignment and the selected clinical characteristics. tSNPs with significant genotype tests (rs4803455, rs4803457, and rs10739778) were evaluated.

Neither average SBP less than 20 weeks' gestation ($H(2)$ = 0.155, p = .093), average DBP less than 20 weeks ($H(2)$ = 0.092, p = .955), or pre-pregnancy BMI ($H(2)$ = 1.360, p = .507) were significantly different between rs4803455 genotype groups. Average SBP less than

20 weeks' gestation ($H(2) = 0.272$, p = .873), average DBP less than 20 weeks ($H(2) = 0.311$, p = .856), and pre-pregnancy BMI ($H(2) = .907$, p = .635) were not significantly different between rs4803457 genotype groups. For rs10739778, average SBP less than 20 weeks gestation ($H(2) = 1.82$, p = .404), average DBP less than 20 weeks ($H(2) = 1.29$, p = .526), and pre-pregnancy BMI ($H(2) = .1.57$, p = .458) were not significantly different between the genotype groups. Average SBP in labor and average DBP in labor were significantly associated with rs4803455, rs4803457, and rs1039778 (rs4803455: SBP- $H(2) = 5.873$, p = .053, DBP- $H(2) = 6.582$, p = .037; rs4803457: SBP- $H(2) = 6.537$, p = .038, DBP- $H(2) = 6.362$, p = .042; rs10739778: SBP- $H(2) = 7.174$, p = .028, DBP- $H(2) = 7.090$, p = .029).

3.5 DISCUSSION

The *ENG* gene codes for a trans-membrane receptor that influences systemic endothelial function (Jerkic et al., 2004; Toporsian et al., 2005) and the degree of placental implantation/remodeling of uterine spiral arteries during pregnancy (Caniggia et al., 1997; Mano et al., 2011). Women with preeclampsia have increased levels of *ENG* mRNA in the placenta and/or blood (Farina et al., 2008; Farina et al., 2010; Nishizawa et al., 2007; Purwosunu et al., 2008; Purwosunu, Sekizawa, Okazaki, et al., 2009; Purwosunu, Sekizawa, Yoshimira, et al., 2009; Sekizawa et al., 2012; Sitras et al., 2009; Toft et al., 2008; Tsai et al., 2011; Venkatesha et al., 2006) along with increased protein levels of soluble ENG (sENG) in the maternal circulation throughout pregnancy (Rana et al., 2007). Because genetic variation could impact the level of ENG transcription/translation and/or protein structure/function, the purpose of this case-control

candidate gene association study was to examine the association between *ENG* pathway genetic variation and the development of preeclampsia. Using iPLEX® and TaqMan® technologies, we evaluated 47 tSNPs and 2 potentially functional SNPs across five *ENG* pathway candidate genes. We found that genetic variation in *ENG* (rs10121110 and rs11792480) and *TGFβR2* (rs6550005) was associated with susceptibility to/protection from preeclampsia in white women. In *ENG*, rs10121110 and rs11792480 are located next to each other, with approximately 4000 bases between them (Figure 6). Our data further suggest that genetic variation in *TGFβ1* (excluding rs8179181- not genotyped), *TGFβR1*, and *ALK1* is not associated with the development of preeclampsia in white women.

There are several potential explanations that could account for why *ENG* (rs10121110 and rs11792480) and *TGFβR2* (rs6550005) genetic variation may be associated with the development of preeclampsia. As depicted in Figure 6, rs10121110 is located in the intronic region between the second and third exons and it tags a genomic region that includes the *ENG* promoter region. The promoter region lacks a TATA and CAAT box, but contains an SP1 site and SMAD binding elements (SBE) needed for the respective binding of SP1 and Smad transcription factors (Botella, Sánchez-Elsner, Rius, Corbí, & Bernabéu, 2001). Given the location of rs1012110, it is possible rs1012110 is capturing an association that represents a promoter variant that impacts transcription factor access/binding (e.g., SP1 and/or SMAD) and subsequently influences transcription and translation of *ENG*.

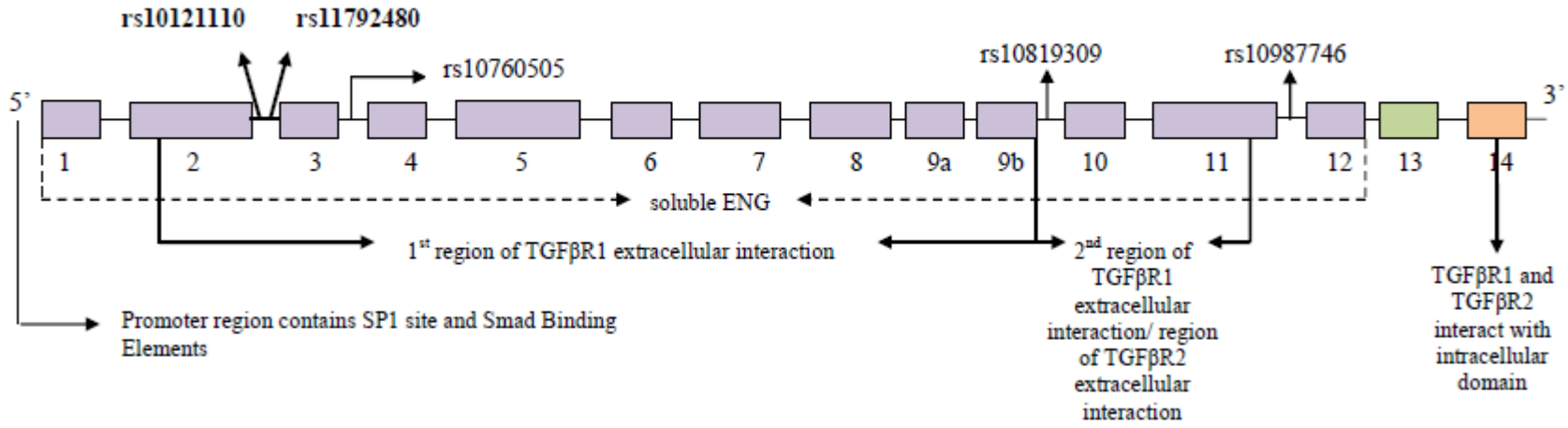


Figure 6. *ENG* Gene Structure

Extracellular domain exons are shaded purple. The transmembrane domain exon is shaded green. The intracellular domain exon is shaded orange. tSNPs with significant allele &/or genotype tests are bolded. Illustration modified from Bosler, Richards, George, Godmilow, & Ganguly, 2006. Information from Guerrero-Esteo et al., 2002 and UCSC Genome Browser (Fujita et al., 2011) was utilized to create this illustration. Sequence and tSNP location determined by UCSC Genome Browser (Fujita et al.), information about promoter region obtained from Botella et al., 2001, and information on soluble endoglin obtained from Hawinkles et al., 2010

Research has shown that knockdown of *ENG* in human extravillous trophoblast (EVT) cell line via short hairpin RNA specific for *ENG* (Mano et al., 2011) or knockdown of first trimester human trophoblast villous explants via antisense endoglin nucleotides (Caniggia et al., 1997) improves the invasive capacity of EVTs, which are essential to uterine spiral artery remodeling in pregnancy (Caniggia et al.; Mano et al.). As such, a promoter variant in *ENG* could contribute to the elevated expression of placental *ENG,* reducing the invasive capacity of EVTs, and explaining the observed increase in ENG noted in preeclampsia. This elevation in placental *ENG* expression could therefore inhibit EVT invasion of uterine spiral arteries, resulting in the shallow implantation and reduced placental perfusion that is observed in preeclampsia. Hypothetically, to compensate for excess membrane-bound placental ENG receptors, matrix metalloproteinase-14 (MMP-14) could cleave the excess receptors in order to improve uterine spiral artery invasion and remodeling. Consequently, MMP-14 cleavage of membrane-bound ENG results in the release of sENG (Kaitu'u-Lino et al., 2012), which is elevated in the maternal circulation of women with preeclampsia and has been suggested to cause endothelial dysfunction (e.g., vascular tone abnormalities) (Venkatesha et al., 2006). Future studies examining the *ENG* promoter, its transcription factors, and *MMP-14* will help us to better understand the mechanisms driving the observed differences in ENG and sENG.

Intronic tSNPs rs10121110 and rs11792480 are also located between exons that code for the extracellular domain of the ENG receptor (Figure 6). As part of the TGFβ1 signaling cascade, TGFβR1 interacts with amino acid residues 26-437of ENG's extracellular domain (Guerrero-Esteo, Sánchez-Elsner, Letamendia, & Bernabéu, 2002). It is only through the interaction of ENG and the type 1 and 2 receptors that ENG can gain access to TGFβ1 (Guerrero-Esteo et al.). For that reason, genetic variation within *ENG*'s extracellular domain

could influence ENG's ability to interact with TGFβR1, thereby affecting ENG's access to TGFβ1 and the transmission of TGFβ1 signals. Since TGFβ1 induces *ENG* expression (Mano et al., 2011) and stimulates *ENG* promoter activity (Rius et al., 1998), a genetic variation that impacts the degree of TGFβ1 transmission may also explain the differences in *ENG* expression (mRNA) observed in women with/without preeclampsia. Future studies directed at the examination of the genetic regions tagged by rs10121110 and rs11792480 may provide greater insight into how *ENG* is involved in the development of preeclampsia.

Based on the Human Feb. 2009 (GRCh37/hg19) Assembly of UCSC genome browser, *TGFβR2* tSNP rs6550005 is intronically located between the first two exons. Given rs6550005's proximity to *TFGβR2*'s promoter region, rs6550005 may tag a promoter variant that influences level of *TGFβR2* transcription/translation. Because ENG can only bind TGFβ1 ligand in the presence of the type 1 and 2 signaling receptors (Guerrero-Esteo et al., 2002), alteration in *TGFβR2* transcription and translation could impact the number of TGFβR2 receptors available for ENG interaction and transmission of TGFβ1 ligand signaling.

Our haplotype analysis further supports the association between *ENG* gene variation and the development of preeclampsia. We found that the TACGA haplotype, which houses the risk alleles of each of the independently associated tSNPs (rs10121110, rs11792480) was over-represented in cases. This consistency in findings from the allele, genotype, and haplotype tests increases our confidence in our findings.

Analysis of the association between *ENG* pathway candidate genes and preeclampsia was a more exploratory endeavor in the black sample given the much smaller sample size. Despite this potential lack of power, variation in several candidate genes (*TGFβ1*, *TGFβR1*, and *TGFβR2*) was associated with development of preeclampsia in black women. *TGFβR2* tSNP

rs6550005 was the only tSNP significantly associated with preeclampsia in both blacks and whites. The remaining associations in *TGFβR2* (intronic tSNPs rs1346907 and rs877572), *TGFβ1* (intronic tSNPs rs4803455 and rs4803457), and *TGFβR1* (intronic tSNP rs107399778) were only found in the black sample. Our data further suggest that variation in *ENG* and *ALK1* may not be associated with preeclampsia development in black women.

Mechanistically, these results suggest that the pathway's involvement in preeclampsia development may differ in blacks and whites. The study of *TGFβ1* overexpression in black hypertensive subjects compared to white hypertensive subjects by Suthanthiran et al. (2001) provides support for this suggested etiologic difference. Operating under the premise that *TGFβ1* over-expression in blacks may be associated with the increased incidence/prevalence of hypertension; Suthanthiran et al. compared TGFβ1 mRNA and protein levels in hypertensive black and hypertensive white subjects. TGFβ1 protein levels were significantly higher in hypertensive blacks compared to hypertensive whites ($p < .001$), suggesting that different physiologic and genetic differences drive the hypertensive phenotype in blacks.

Like Sunthanthiran et al. (2001), *TGFβ1* variation was associated with the hypertensive disorder of preeclampsia in our black sample. Moreover, average blood pressure in labor (SBP and DBP) was significantly associated with *TGFβ1* tSNP rs4803455 and tSNP rs4803457 genotypes along with *TGFβR1* tSNP rs10739778 genotypes in our cohort. Given these associations, it is feasible to hypothesize that these tSNPS could tag genetic variants in *TGFβ1* and/or *TGFβR1* that alter protein function and/or structure, affect transcription/translation, or affect the interaction between TGFβ1 and TGFβR1. Such genetic perturbations also lead to the following question: Is *TGFβ1* a common risk factor for preeclampsia and later life cardiovascular disease (e.g., hypertension) in black women? Further investigation with a larger

sample size is needed to answer this question, verify these findings, and provide greater insight into the mechanisms of these candidate genes in black women with preeclampsia.

To date, only one additional study has looked at the association between *ENG* and preeclampsia separately in white and black women. Srinivas, Morrison, Andrela, and Elovitz (2010) used a pre-designed IBCv2 array (Illumina Inc, San Diego, CA) to examine angiogenic pathway genes. Of the five candidate genes examined in our study, only *ENG* was included their array. Srinivas et al. (2010) did not find any significant associations between *ENG* tSNPs and preeclampsia in white women (32 cases/85 controls) and black women (184 cases/305 controls). This is in contrast to the current study in which we found two *ENG* tSNPs (rs10121110 and rs rs11792480) to be significantly associated with preeclampsia in white women. Ultimately, the lack of association in the study by Srinivas et al. is likely driven by lack of power in the white subgroup. Like Srinivas et al., we did not find any tSNPs in *ENG* to be significantly associated with preeclampsia in the black subgroup; and their evaluation did not include the genes we found to be associated with preeclampsia in our black subjects.

There were several limitations associated with this case-control candidate gene association study. First, *TGFβ1* tSNP rs8179181 and *TGFβR2* tSNP rs3087465 could not be genotyped despite multiple attempts with iPLEX® and TaqMan® platforms likely due to the fact that these tSNPs were multiallelic (4 alleles & 3 alleles). Without this information, our ability to fully evaluate the genetic variability of *TGFβ1* and *TGFβR2* was impaired. Second, the black subgroup may have been underpowered and associations between the candidate genes and preeclampsia may have been missed. Third, tSNPs were selected for Caucasian ancestry. Because linkage disequilibrium in the candidate genes may be different for those of African ancestry, the haploblocks tagged by tSNPs selected for Caucasian ancestry may be different than

haploblocks tagged by tSNPs selected based on African Ancestry, resulting in decreased informativeness in blacks. Fourth, our study was limited to white and black women. Results generated for these subgroups may not be generalizable to other ancestries. Fifth, we evaluated our logistic regression results with a more stringent p-value criterion ($\alpha = .01$) in order to account for multiple testing. Although some results were significant at the $p < .05$ level, they became non-significant at the $p < .01$ level. This change in significance may have decrease our interest in the association, but our sample may not have had enough power to detect these associations at the $\alpha = .01$ level.

3.6 CONCLUSION

Our study demonstrated that *ENG* pathway genetic variation is associated with preeclampsia in white and black women. Our results further suggest that the pathway's involvement in preeclampsia differs in whites and blacks, with *ENG* and *TGFBR2* being associated in whites and *TGFβ1*, *TGFβR1*, and *TGFβR2* being associated in blacks. However, replication of these results is needed to confirm these findings, especially in the black subgroup because of its small sample size. Moreover, because these significant associations between *ENG* pathway tSNPs and preeclampsia are likely not causative, further examination of the genomic regions (e.g., promoter region of *ENG*) tagged by these polymorphisms would further improve our understanding of this pathway's role in preeclampsia.

APPENDIX A

UNIVERSITY OF PITTSBURGH INSTITUTIONAL REVIEW BOARD APPROVAL LETTERS

University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
http://www.irb.pitt.edu

Memorandum

To: Dr. Mandy Bell
From: Sue Beers PhD, Vice Chair
Date: 12/2/2009
IRB#: PRO09110136
Subject: Genomics of Endoglin Pathway in Preeclampsia (GEPP)

The University of Pittsburgh Institutional Review Board reviewed and approved the above referenced study by the expedited review procedure authorized under 45 CFR 46.110. Your research study was approved under:
45 CFR 46.110.(7)

This study is supported by the following federal grant application(s):
F31NR011379 Genomics of Endoglin Pathway in Preeclampsia (GEPP)

Approval Date: 12/1/2009
Expiration Date: 11/30/2010

For studies being conducted in UPMC facilities, no clinical activities can be undertaken by investigators until they have received approval from the UPMC Fiscal Review Office.

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least one month prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

University of Pittsburgh
Institutional Review Board

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(412) 383-1508 (fax)
http://www.irb.pitt.edu/

Memorandum

To: Ms. Mandy Bell
From: Christopher Ryan, PhD, Vice Chair
Date: 9/16/2010
IRB#: REN10090025 / PRO09110136
Subject: Genomics of Endoglin Pathway in Preeclampsia (GEPP)

Your renewal for the above referenced research study has received expedited review and approval from the Institutional Review Board under:
45 CFR 46.110.(7) characteristics/behaviors

Please note the following information:

Approval Date: 9/15/2010
Expiration Date: 9/14/2011

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month** prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

University of Pittsburgh
Institutional Review Board

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Memorandum

To: Mandy Bell
From: Sue Beers, PhD, Vice Chair
Date: 8/3/2011
IRB#: REN11080007 / PRO09110136
Subject: Genomics of Endoglin Pathway in Preeclampsia (GEPP)

Your renewal for the above referenced research study has received expedited review and approval from the Institutional Review Board under: **This approval is for analysis of data only.**

45 CFR 46.110.(7) characteristics/behaviors

Please note the following information:

Approval Date: 8/3/2011
Expiration Date: 8/2/2012

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month** prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

APPENDIX B

MANUSCRIPT #1: A HISTORICAL OVERVIEW OF PREECLAMPSIA-ECLAMPSIA

JOGNN

PRINCIPLES & PRACTICE

A Historical Overview of Preeclampsia-Eclampsia

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Keywords
preeclampsia-eclampsia
historical overview
practice trends

ABSTRACT

Preeclampsia is a hypertensive, multisystem disorder of pregnancy whose etiology remains unknown. Although management is evidence-based, preventative measures/screening tools are lacking, treatment remains symptomatic, and delivery remains the only cure. Past hypotheses/scientific contributions have influenced current understanding of preeclampsia pathophysiology and guided management strategies and classification criteria. To provide insight into how past hypotheses/scientific contributions have shaped current practice trends, this article provides a historical overview of preeclampsia-eclampsia.

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Preeclampsia is a hypertensive, multisystem disorder of pregnancy that significantly contributes to maternal and fetal/neonatal morbidity and mortality (American College of Obstetricians and Gynecologists [ACOG], 2002; National Heart, Lung, and Blood Institute [NHLBI] National High Blood Pressure Education Program, 2000). At present, the etiology of preeclampsia remains unknown. As a result, preventative measures and screening tools are lacking, treatments are directed at the management of overt clinical manifestations, and delivery remains the only definitive cure (ACOG; NHLBI National High Blood Pressure Education Program; Norwitz & Repke, 2009).

Despite our inability to identify preeclampsia's etiology, hypotheses and scientific contributions throughout history have influenced our current understanding of preeclampsia pathophysiology. Such contributions are further reflected in current management strategies and classification criteria. To provide insight into how current practice trends have been shaped by past hypotheses and scientific contributions, this article provides a historical overview of preeclampsia-eclampsia from ancient times through present day. Although it is not the intent of this article to provide an all-inclusive historical analysis of primary sources, attention is directed toward an overview of theories on disease causation, treatments, and disease classifications extracted from available primary and reliable secondary sources.

Ancient Times

Theories on Disease Causation

In ancient Greece, between the late 5th and early 4th centuries BC, the Hippocratics subscribed to the theory of the four humors to describe the cause of illness and disease. They believed that the body was made up of four humors (fluids) that included blood, phlegm, yellow bile, and black bile. Health depended on a balance of the humors, and any imbalance in the humors resulted in illness (Demand, 1994; Green, 1985).

The wet and dry theory was used to explain the vulnerability of female physiology to disease (Green, 1985). Women were considered wet while men were considered dry (Green; Trotula of Solerno, 1544/1940). Because a woman's flesh was porous and soft, she was at risk of drawing in too much moisture, resulting in an overabundance of fluids (humors) and subsequent illness (Green).

More specifically, many female ailments were attributed to the wandering womb. Central to this theory was that the uterus was able to physically uproot itself from its seat in the pelvis and travel anywhere within a woman's body in search of satisfaction. Although Hippocrates believed that a dried-up uterus wandered the body in search of moisture, Plato viewed the uterus as an animal that wandered because it was sexually unsatisfied and desired to make children (Thompson, 1999).

Regardless of reason, as the uterus wandered the body, it was capable of wreaking havoc upon the liver, stomach, spleen, lungs (Thompson), and head (Veith, 1965), ultimately leading to disease.

Treatment

Because disease was believed to result from either an imbalance in the four humors, a woman's overly porous skin (Demand, 1994; Green, 1985), or a wandering womb (Thompson,1999), treatments focused on the restoration and maintenance of internal balance and health. As a result, remedies to restore balance included altered diets, purging, and bloodletting. As for methods to maintain balance and health, the Hippocratics believed that a woman needed to be pregnant, lactating, or regularly menstruating. These methods were based on the belief that pregnancy, lactation, and menstruation provided mechanisms for the elimination of excess fluids. During pregnancy, extra blood was used to nourish the growing fetus. During lactation, extra blood was diverted to the breasts where it was converted to milk and eliminated through breast feeding. During menstruation, excess blood was eliminated through menstrual flow (Green).

Disease Classification

Preeclampsia-eclampsia was not formally classified as a disorder of pregnancy during Ancient times. Despite limited knowledge and technology, the writings of this period did demonstrate that the concept of eclampsia was recognized. For example, aphorism XXXI 507 in the Coan Prognosis states that a headache accompanied by heaviness and convulsions during pregnancy is considered bad (Hippocrates, 400 BC/1950).

Middle Ages & the Renaissance

Theories on Disease Causation

During the Middle Ages, medical and scientific progress came to a standstill. Between 400 and 700 AD, Christianity greatly influenced such progress, for Christians were opposed to science and forbade human dissection (Cianfrani, 1960; Graham, 1951). Closing of medical schools at Athens and Alexandria by Byzantium Emperor Justinian in the 6th century further slowed medicine's progression (Cianfrani). Consequently, little original work was accomplished. Instead, individuals such as Oribasius, Aetius of Amida, and Paulus of Aegina focused on the compilation and rewriting of the medical works of their predecessors (Cianfrani; Graham).

By viewing preeclampsia-eclampsia from a historical perspective, nurses gain insight into how past hypotheses and scientific contributions have influenced and shaped current practices.

Between 700 and 1200 AD, Byzantium deteriorated, Christian influence began to decline, and Salernitan (Salerno, Italy) and Arabian influences increased. The first European medical school was opened in Salerno, Italy, and a slightly altered four humors theory emerged. Under this newer theory, it was believed that one humor dominated the other humors to form a new balance. Furthermore, the dominant humor determined an individual's physical and emotional characteristics (Cianfrani, 1960).

During the Renaissance, an acceleration in the progression of medicine resulted from the further waning influence of the church, freedom of the intellect, rearrangement of government and geography, and discovery of printing (Cianfrani, 1960). In 1537, Pope Clement VIII granted permission to teach anatomy by human dissection (Burton, 2005). With this new-found freedom, anatomists and artists such as Jacopo Berengario da Capri (1460–1530), Nicolaus Massa (1499–1569), Leonardo da Vinci (1452–1519), Andreas Vesalius (1514–1564), and Fallopius (1523–1562) were instrumental in more clearly and accurately describing the female reproductive tract. For example, the first accurate description of the tubes and ovaries was provided by Fallopius. He is also credited with naming the placenta and indicating that it was only found in the uterus during pregnancy (Cianfrani).

In the 17th century, medicine continued to gain momentum and men began to enter the field of obstetrics (McMillen, 2003). Born in 1637, Frenchman Francois Mauriceau was one such man whose writings helped to establish obstetrics as a specialty (Speert, 1958). According to McMillen, he was the first to systematically describe eclampsia and to note that primigravidas were at greater risk for convulsions compared to multigravidas. As for the causes of convulsions, Mauriceau attributed convulsions to either abnormalities in lochial flow or intrauterine fetal death. In the case of supressed lochial flow, inflammation, pains in the head, convulsions, suffocation, and death could arise. In the case of intrauterine fetal death, a retained dead fetus gave off foul-smelling and cadaverous humors in the womb, predisposing a woman to convulsions (Mauriceau, 1668/1710).

Treatment

Treatment of disease during the Middle Ages was greatly influenced by Christian beliefs. Remedies prescribed by physicians in Ancient times were often replaced with charms, amulets, faith healing, miracles, and prayers (Cianfrani, 1960; Graham, 1951). However, as time passed and Christian influence waned, remedies similar to those used to treat disease in Ancient times became prominent again. For example, in an attempt to decrease cerebral congestion and prevent eclampsia, Mauriceau recommended two to three phlebotomies during pregnancy (Chesley, 1978, 1984; McMillen, 2003).

Disease Classification

Near the end of the Reniassance, the classification of disease progressed. Gabelchoverus distinguished between four types of epilepsy in 1596, which included epilepsy resulting from the head, stomach, the pregnant uterus, and chilled extremities (Chesley, 1978). However, it wasn't until 1619 that the word *eclampsia* first appeared in Varandaeus' treatise on gynecology (Ong, 2004).

18th Century Through 19th Century

Theories on Disease Causation

In the 18th century, Boissier de Sauvages distinguished eclampsia from epilepsy. Along with the distinction he made in disease classification, de Sauvages offered his views on the cause of convulsions. He believed that convulsions resulted from nature trying to free the organism of any morbid element (Temkin, 1971).

Theories on disease causation continued to be proposed and thoroughly discussed in the writings of 19th century physicians. In his work titled *Introduction to the Practice of Midwifery*, Dr. Thomas Denman (1821) focused much attention on the labors affected by convulsions. Although Denman attributed convulsions to certain customs and manners associated with living in large cities and towns, he noted that the greatest risk of convulsions came from the uterus. According to Denman, as the uterus expanded with pregnancy, greater pressure was placed upon the descending blood vessels. Such an increase in pressure lead to the regurgitation of blood in the head and resulted in an overload of the cerebral vessels and subsequent convulsions (Denman).

In his 1849 work, *Parturition and the Principles and Practice of Obstetrics*, Dr. William Tyler Smith challenged the theory of cerebral congestion, for he believed that pregnancy was a state of increased fullness in circulation. Given that contractions during the second stage of labor normally interfered with the circulation of blood, he believed that more cases of convulsions would be observed if such congestion caused convulsions. In contrast, Smith attributed puerperal convulsions to several other causes: (a) any mechanical or emotional stimulus applied in excess to the spinal center; (b) bloodletting; (c) variations in the wind, temperature, and other atmospheric changes; (d) irritation of the uterus, uterine passages, intestinal canal, and the stomach; and (e) "toxic" elements. As for Smith's theory on "toxic" elements, he believed that preservation of health during pregnancy depended on the exponential increase in the elimination of wastes (e.g., secretions of the bowels) and debris from the maternal and fetal systems. Failure to do so resulted in a "toxemia" in which morbid elements accumulated in the blood causing irritation to the nervous center (Smith, 1849).

Treatment

Bloodletting remained a staple in the prevention and treatment of preeclampsia-eclampsia during the early 1800s. The amount and frequency of bloodletting depended on the strength of the patient and symptom severity. Bleeding from the arm was attempted initially, but if convulsions continued, bleeding was repeated. In some cases the jugular vein or temporal artery were opened in an attempt to stop convulsions (Denman, 1821).

The use of opiates, warm baths, splashing of the face with cold water, and hastening of delivery were also recommended by Denman. Opiates were used to decrease irritability of the female constitution. If bloodletting and opiates failed, splashing of cold water to the patient's face or placement of the patient in a warm bath were attempted. In cases where all treatments were unsuccessful, the physician had to choose between either hastening delivery or allowing natural labor to ensue. Per Denman, hastening of delivery was only to be attempted when a woman was physiologically ready (completely dilated, ruptured membranes, and fetus descended) because intervention in the early stage of labor increased maternal mortality (Denman, 1821).

When the theory of disease causation shifted to the toxin theory in the late 1800s, treatments were targeted at the elimination of overabundant toxins. Those who believed that preeclampsia-eclampsia was caused by meat toxins restricted the consumption of meat and prescribed diets of fruits, vegetables, and milk products (Chesley, 1978). With the recognition of the preeclamptic state, women with headaches and edema of the superior extremities were admitted to lying-in hospitals where they

underwent treatments such as bleeding and purging to prevent convulsions (Johns, 1843).

Disease Classification

After the introduction of the word *eclampsia*, Bossier de Sauvages (1739) differented eclampsia from epilepsy (Chesley, 1978; Friedlander, 2001). Eclampsia was acute in nature because convulsions resolved once the precipitating event was removed. Epilepsy was chronic in nature because convulsions recurred over time (Friedlander). Furthermore, eclampsia was not restricted to pregnancy. Severe hemorrhage, various sources of pain, vermicular infestations, and eclampsia associated with pregnancy were several species of eclampsia noted by de Sauvages (Chesley, 1978).

At the end of the 18th century and through the 19th century, the classification of preeclampsia-eclampsia continued to become more refined as the classic signs and symptoms of preeclampsia-eclampsia became more readily recognized. In 1797, Demanet noted a connection between edematous women and eclampsia (Chesley, 1978) whereas John Lever discovered albumin in the urine of eclamptic women in 1843 (Thomas, 1935). The connection between premonitory symptoms during the later months of pregnancy and the development of puerperal convulsions was also recognized in 1843 by Dr. Robert Johns. These premonitory symptoms included headache, temporary loss of vision, severe pain in the stomach, and edema of the hands, arms, neck, and face (Johns, 1843). In 1897, Vaquez and Nobecourt were credited with the discovery of eclamptic hypertension (Chesley, 1978). As a result of these contributions, the concept of the preeclamptic state was recognized. Physicians were now aware that the presence of edema, proteinuria, and headaches should raise concern about the possibility of convulsions (Sinclair & Johnston, 1858).

20th Century

Theories on Disease Causation

Although researchers in the 20th century failed to uncover the etiology of preeclampsia, much progress was made in the understanding of pathophysiological changes associated with its development. In the 1960s, several groups described dramatic differences in placental physiology between placentas from pregnancies affected by preeclampsia versus placentas from pregnancies unaffected by preeclampsia. Through the examination of placental bed biopsies, it was discovered that placental trophoblast cells failed to adequately invade maternal spiral arteries and convert the arteries from small muscular vessels into large, low-resistant vessels in preeclampsia. With the lack of spiral artery conversion, arterial lumen diameter and distensibility was limited, resulting in restricted blood flow to the placenta and growing fetus (Brosens, Robertson, & Dixon, 1967, 1972; Gerretsen, Huisjes, & Elema, 1981; Kong, De Wolf, Robertson, & Brosens, 1986).

Although these findings were instrumental in laying the groundwork for the current understanding of preeclampsia-elcampsia, not all theories or scientific discoveries have readily been accepted by the scientific community. Published in the *American Journal of Obstetrics and Gynecology* in 1983, the Hydatoxi lualba (parasitic worm) theory of preeclampsia was one such theory quickly refuted by the scientific community. Under this theory, it was posited that the development of preeclampsia-eclampsia may be associated with the presence of a worm-like organism. Specimens collected from women with preeclampsia-eclampsia, including peripheral circulating blood, bloody fluid on the maternal surface of the placenta, and umbilical cord blood, were found to be positive for Hydatoxi lualba (Lueck, Brewer, Aladjem, & Novotny, 1983). However, several other research groups demonstrated that starch powder from gloves, cellulose debris from common laboratory paper products, and alterations in staining technique produced the same characteristic worm-like organisms (Papoutsis, Irwin, Curry, & Zuspan, 1983; Sibai & Spinnato, 1983), which lead to refutation of the theory.

Unlike the parasitic worm theory, the theory posited by Roberts and colleagues in 1989 continues to guide research related to preeclampsia-eclampsia etiology. Dr. Roberts and colleagues posited that preeclampsia represented an endothelial disorder. Drawing on past work that associated preeclampsia with shallow trophoblast invasion and subsequent reduction in placental perfusion, they hypothesized that the ischemic placenta released a damaging factor(s) into the maternal circulation. Although factor identity was unknown, the circulating factor was hypothesized to have caused endothelial dysfunction and would lead to activation of the coagulation cascade, blood pressure abnormalities, and loss of fluid from the intravascular space (e.g., proteinuria) (Roberts et al., 1989).

Treatment

At the end of the 19th century and into the beginning of the 20th century, two very diverse approaches were used in the treatment of eclampsia. According

to Chesley, physicians in Germany and the Netherlands advocated for aggressive management (e.g., prompt abdominal or vaginal Cesarean section), but the associated maternal mortality rates were extremely high. As a result, a more conservative management gained popularity and was widely used up until the 1930s (Chesley, 1978, 1984). Physician pioneers of this conservative method included Tweedy of Dublin and Stroganoff of Russia (Speert, 1958).

Tweedy's rationale for conservative management (as cited in Speert, 1958) was rooted in the belief that hastening of labor and delivery increased the occurrence of convulsions through the induction of reflex stimulation. Physicians were to abstain from vaginal examinations, abdominal palpation, massage of the kidneys, cold blasts of air, and the dilatation of the cervix in an effort to mitigate the risk of reflex stimulation. In contrast, his management plan revolved around patient sedation and included large doses of morphine. However, if a patient went into labor, he believed that the application of forceps was permissible given that the os would safely permit their application (Speert).

Because convulsions disrupted the functions of the heart, lungs, kidneys, and liver, Stroganoff's main objective was to eliminate convulsions (Speert, 1958). He treated the eclampsia, ignored the pregnancy, and waited for natural onset of labor. All examinations and treatments were performed under light anesthesia, and sensory stimuli were reduced by keeping the patient's room dark and quiet (Chesley, 1984). Morphine and chloral hydrate were administered to keep patients sedated and to decrease frequency of convulsions. To restore respiratory function, oxygen was administered. To restore cardiac function, digitalis was administered if the pulse was found to be rapid and weak after a seizure (Chesley, 1984; Speert). Labor was to progress naturally and once a woman's cervix had dilated to 6 cm, the membranes were artificially ruptured (Chesley, 1984).

In addition to the diverse approaches to manage preeclampsia-eclampsia in the 20th century, the use of magnesium sulfate was introduced. Although a mainstay of current treatment, it was not until 1906 that Horn first used magnesium sulfate to manage preeclampsia-eclampsia (Chesley, 1984). During the 1920s, the parenteral use of magnesium sulfate in the treatment of preeclampsia-eclampsia was popularized by Lazard and Dorsett (Chesley, 1984), for Dr. Lazard's work (as cited in Gabbe, 1996) demonstrated that treatment with intravenous magnesium sulfate was efficacious and safe.

Since the 1960s, few alterations have been made in the management of preeclampsia. Such consistency can be observed by studying the obstetrical textbooks used to educate students within the health care fields. Routine prenatal care (e.g., routine blood pressure measurement, urinalysis, maternal weight) remained a hallmark surveillance method because early signs and symptoms of preeclampsia may not be readily recognized by pregnant women. Once diagnosed with preeclampsia, management consistently included hospitalization, frequent blood pressure measurement and weighing, urinalysis, bed rest, fetal surveillance, and assessment of maternal headache, blurry vision, and epigastric pain. With fulminating preeclampsia or the development of eclampsia, magnesium sulfate and antihypertensives were administered over the later part of the 20th century to prophylactically prevent or manage convulsions and acute hypertension, respectively. Ultimately, the decision to proceed with a vaginal delivery or Cesarean section depended on a myriad of factors, including gestational age, condition of the cervix, and maternal and fetal condition (Eastman & Hellman, 1966; Hibbard, 1988; Pritchard & MacDonald, 1976).

Disease Classification

As with the treatment of preeclampsia, review of obstetrical textbooks provides insight into the changes made in preeclampsia-eclampsia disease classification throughout the 20th century (Table 1). According to Chesley (1978), the "preeclamptic" state was not included in textbooks until 1903. Furthermore, restriction of preeclampsia-eclampsia to the obstetric definition was not observed until 1961 (Chesley, 1978).

In the 13th edition of *Williams Obstetrics* (1966), preeclampsia-eclampsia fell under the category of the toxemias of pregnancy. According to the classification set forth by the American Committee on Maternal Welfare, toxemias of pregnancy included acute toxemia of pregnancy (preeclampsia and eclampsia), chronic hypertensive disease with pregnancy, and unclassified toxemia. Criteria for the diagnosis of preeclampsia included the presence of hypertension, edema, or proteinuria after the 24th week of gestation. Moreover, this classification required that a woman must meet only one of the three criteria to be diagnosed with preeclampsia (Eastman & Hellman, 1966).

Table 1: Progression of Preeclampsia-Eclampsia Classification During the 20th Century

Year & Citation	Milestone
1903 Chesley (1978)	"Pre-eclamptic state" included in textbooks
1961 Chesley (1978)	Preeclampsia-eclampsia restricted to the obstetric definition

Obstetrical Textbook Publication Year & Citation	Terminology	Classification Description
1966 Eastman and Hellman (1966)	Toxemias of pregnancy	A. Acute toxemia of pregnancy (preeclampsia and eclampsia); chronic hypertensive disease with pregnancy; unclassified toxemia B. Preeclampsia diagnostic criteria: presence of hypertension, edema, or proteinuria after 24 weeks gestation
1976 Pritchard and MacDonald (1976)	Hypertensive disorders of pregnancy	A. "Toxemias of pregnancy" replaced with "hypertensive disorders of pregnancy" B. Preeclampsia diagnostic criteria: development of hypertension with proteinuria, edema, or both commencing after 20 weeks gestation
1988 Hibbard (1988)	Pregnancy induced hypertension	A. Under the classification of hypertensive disorders of pregnancy, preeclampsia was further grouped under "pregnancy-induced hypertension," which also included hypertension that developed during pregnancy excluding the features of preeclampsia B. Preeclampsia diagnostic criteria: mild to moderate preeclampsia—presence of hypertension and edema; severe preeclampsia—presence of hypertension and proteinuria with or without edema or cerebral or visual disturbances after 20–24 weeks gestation

In the 15th edition of *Williams Obstetrics* (1976), the term *toxemia of pregnancy* was replaced with *hypertensive disorders of pregnancy*. The Committee on Terminology of the American College of Obstetricians and Gynecologists recommended new classifications. The new classification of preeclampsia included the development of hypertension with proteinuria, edema, or both commencing after 20 weeks gestation (Pritchard & MacDonald, 1976).

In Hibbard's 1988 text titled *Principles of Obstetrics*, the classification of preeclampsia underwent yet another revision. Although preeclampsia fell under the hypertensive disorders of pregnancy classification, it was further grouped under the term *pregnancy-induced hypertension*. In addition to preeclampsia, hypertension that developed during pregnancy excluding the features of preeclampsia was also grouped under the term *pregnancy-induced hypertension*. Mild to moderate preeclampsia was classified as the presence of hypertension and edema whereas severe preeclampsia was classified as the presence of hypertension and proteinuria with or without edema or cerebral or visual disturbances (Hibbard, 1988).

21st Century

Theories on Disease Causation

At present, the scientific community has failed to uncover the etiologic mechanisms responsible for the development of preeclampsia-eclampsia. As evidenced by the many review articles published in

Although preeclampsia-eclampsia management is guided by the best available evidence, preventative measures/ screening tools are lacking, treatments are symptom based, and delivery remains the only cure.

the scientific literature, the theories on disease causation are numerous and diverse. Such theories are related to mechanisms involving oxidative stress, immunologic intolerance between the fetoplacental unit and maternal tissue, and angiogenic imbalance (Leeman & Fontaine, 2008). For example, the endoglin protein, which is involved in regulation of placental trophoblast differentiation/invasion of the uterus (Caniggia, Taylor, Ritchie, Lye, & Letarte, 1997) and maintenance of vascular tone (Jerkic et al., 2004; Toporsian et al., 2005), represents an antiangiogenic factor potentially involved in preeclampsia development given that placental and blood pressure abnormalities are observed in preeclampsia.

Regardless of the mechanism, a two-stage model of preeclampsia has been developed to provide a guiding framework for scientists in their search of disease causation (Hladunewich, Karumanchi, & Lafayette, 2007; Roberts & Gammill, 2005; Roberts & Hubel, 2009). For an in-depth review, consultation of the latest model iteration presented by Roberts and Hubel is recommended. Briefly, the model proposes that reduced placental perfusion (Stage 1), secondary to abnormal implantation and subsequent vascular remodeling, interacts with maternal constitutional factors (genetic, behavioral, and environmental) to produce the maternal syndrome (Stage 2) of preeclampsia. The systemic maternal syndrome is characterized by reduced perfusion brought about by vasospasm and activation of the coagulation cascade with the formation of occlusive microthrombae. This leads to reduced perfusion to multiple organs, hypertension, proteinuria, and loss of fluid from the intravascular space (Roberts & Gammill).

Although it was initially thought that maternal factors only interacted with reduced placental perfusion to produce the maternal syndrome (Stage 2), it is now believed that maternal factors may be involved in the genesis of reduced placental perfusion (Stage 1). It has been further hypothesized that the linkage between Stage 1 and 2 may involve multiple factors, whose constitution may vary from individual to individual. For example, recent suggestions indicate that the placentally derived "toxins" (e.g., cytokines, antiangiogenic factors, and syncytiotrophoblast microparticles) thought to link Stage 1 and 2 may not be pathogenic. In contrast, it has been proposed that placental factors are appropriately released by the fetal/placental unit to increase nutrient availability but are not tolerated by some women who develop preeclampsia (Roberts & Hubel).

Treatment

In an era of evidenced-based practice, the standardized care of women affected by preeclampsia-eclampsia is guided by the best available evidence. Based on the National High Blood Pressure Education Program (2000) Working Group on High Blood Pressure report, the American College of Obstetricians and Gynecologists' (ACOG, 2002) most recent practice bulletin indicates that current management of preeclampsia-eclampsia is reflective of past treatments. Although ACOG's bulletin was published 8 years ago, a more current review of evidence-based information on the management of preeclampsia further demonstrates that the mainstay of treatment has remained consistent (Norwitz & Repke, 2009). Despite consistent, evidenced-based management strategies, the etiology of preeclampsia remains unknown. As a result, effective preventative measures and screening tools are lacking, treatments remain directed at the management of overt clinical signs and symptoms, and the only definitive cure remains delivery (ACOG; NHLBI National High Blood Pressure Education Program, 2000; Norwitz & Repke). However, it is likely that our current evidence-based practices will continue to evolve as we gain a more comprehensive understanding of preeclampsia-eclampsia.

Diagnosis of preeclampsia continues to be based on prenatal blood pressure and urinary protein measurements and initial disease severity is evaluated with laboratory testing. Fetal well-being is monitored via fetal movement counts, nonstress tests, and biophysical profiles. Blood pressure and urine protein measurements, follow-up laboratory testing, and assessment of additional signs/symptoms suggestive of preeclampsia (headache, blurred vision, right upper quadrant or epigastric pain) are used to monitor maternal well-being. The timing and type of delivery ultimately depends on gestational age, maternal and fetal conditions, and the severity of preeclampsia. As for pharmacologic management, magnesium sulfate is administered during labor, delivery, and postpartum to prevent convulsions in women with preeclampsia or to deter recurrent convulsions in women with eclampsia. In addition, antihypertensive therapy (e.g., hydralazine or labetalol) is administered to treat acute

hypertensive episodes (NHLBI National High Blood Pressure Education Program, 2000; Norwitz & Repke, 2009).

Disease Classification

In 2000, the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy published a report with revisions to preeclampsia-eclampsia classification criteria. Preeclampsia is currently classified as a pregnancy-specific syndrome characterized by the presence of new-onset hypertension in a previously normotensive woman after 20 weeks gestation with proteinuria. Blood pressure (BP) criteria include a systolic BP $>$ 140 mm Hg or a diastolic BP $>$ 90 mm Hg. *Proteinuria* is defined as urinary excretion of $\geq$ 0.3 g of protein in a 24-hour specimen, which correlates with a random $\geq$ 1+urine dipstick in the absence of a urinary tract infection. The presence of edema was dropped from the diagnostic criteria because many pregnant women with normal pregnancies develop edema.

Furthermore, eclampsia is classified as the presence of seizures, nonattributable to other causes, in a woman diagnosed with preeclampsia. For additional information on classification of other hypertensive disorders of pregnancy (e.g., gestational hypertension or chronic hypertension, and preeclampsia superimposed on chronic hypertension), a review of the Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy is recommended (NHLBI National High Blood Pressure Education Program, 2000).

Conclusion: Nursing Implications

This historical overview provides present-day nurses with a broadened perspective of preeclampsia-eclampsia related to theories on disease causation, evolution of treatments, and refinement of disease classification. With such a perspective, nurses gain insight into how past hypotheses and scientific contributions have influenced and shaped current practices. Although the etiology of preeclampsia remains unknown, ongoing research has vastly improved our understanding of preeclampsia over the years and continues to guide evidence-based management of women with preeclampsia and refine its classification. However, as we continue to gain a more comprehensive understanding of preeclampsia, it is likely that the current practices used to care for women with preeclampsia-eclampsia will also evolve to reflect the most up-to-date scientific evidence related to preeclampsia etiology and treatment.

Preeclampsia is currently classified as a pregnancy-specific syndrome characterized by new-onset hypertension in a previously normotensive woman after 20 weeks gestation with proteinuria.

As in the past, the current role of nurses in the management of preeclampsia-eclampsia continues to revolve around the protection of maternal–fetal well-being and optimization of positive health outcomes. Given that effective preventative measures and screening tools are presently lacking, routine nursing assessments of the signs/symptoms indicative of preeclampsia-eclampsia remains critical to the detection, monitoring, and effective management of preeclampsia-eclampsia. Nurse-led patient education and the provision of a supportive environment are also essential to the optimal management of preeclampsia-eclampsia. Active participation in one's care can be promoted through nurse-led education related to self-monitoring of fetal activity and maternal symptoms (e.g., headaches, blurred vision, epigastric pain). Furthermore, review of the rationale behind all tests (e.g., laboratory analysis, nonstress test) and treatments (e.g., magnesium sulfate, antihypertensive) keeps patients informed and may help to alleviate stress and anxiety during an emotionally and physically trying time. Ultimately, individually tailored and compassionate nursing care of women with preeclampsia-eclampsia will serve to enhance the well-being of mother and baby.

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APPENDIX D

MANUSCRIPT #2: A SYSTEMATIC REVIEW OF ENDOGLIN GENE EXPRESSION IN PREECLAMPSIA

Citation per SAGE publications:

Bell, M. J. & Conley, Y. P. (2011). A systematic review of endoglin gene expression in preeclampsia. *Biological Research for Nursing.* Advance online publication. doi: 10.1177/1099800411420133.

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A Systematic Review of Endoglin Gene Expression in Preeclampsia

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Abstract

Objective: To synthesize scientific literature that addresses the role of endoglin (ENG) gene expression in preeclampsia (PE). Data sources: A literature search of PubMed and Ovid MEDLINE was conducted using the keywords *endoglin, gene,* and *preeclampsia*. Restrictions included English language and humans. Additional articles were identified/selected for evaluation via PubMed e-mail updates (keywords: *endoglin* and *preeclampsia*) and review of article reference lists obtained from the search. Study selection: The initial 14 abstracts retrieved from the literature search were reviewed and 9 studies were selected for evaluation. Review articles and studies not addressing ENG expression (messenger RNA [mRNA] level) in the context of PE were excluded. An additional six articles were selected from PubMed e-mail updates and reference lists. Data extraction: Data related to study objective, design, setting, subject information, phenotype, tissue type, data collection method, statistics, and results were extracted. Data synthesis: Regardless of PE definition, ancestral background, gene expression analysis method, tissue type, and time of specimen collection, endoglin appears to play a role in PE development. Moreover, results suggest that a variety of biological mechanisms have the ability to modulate ENG expression in PE, demonstrating the potential complexity associated with endoglin's role in PE. Conclusions: This review article is the first to systematically synthesize evidence related to ENG expression in PE. Findings can be utilized to design future studies that (a) address methodological limitations observed in the reviewed studies and (b) specifically examine why ENG expression levels are altered and address mechanisms explaining how these alterations are involved in PE development.

Keywords

preeclampsia, endoglin, gene

Preeclampsia (PE) is a hypertensive, multisystem disorder of pregnancy that significantly impacts maternal and fetal/neonatal health (National Heart, Lung, and Blood Institute [NHLBI] National High Blood Pressure Education Program, 2000). Classified as new-onset hypertension and proteinuria after 20 weeks' gestation in a previously normotensive woman, PE complicates 3–5% of pregnancies (Roberts & Cooper, 2001) and is estimated to cost the United States $7 billion annually (Preeclampsia Foundation, 2000–2010). Although it is believed that PE development involves (a) reduced placental perfusion secondary to abnormal placentation and (b) the maternal syndrome characterized by systemic endothelial dysfunction (Roberts & Hubel, 2009), the factors/mechanisms responsible for these aberrations remain unknown. Several investigations, however, have identified endoglin as a potential factor in the genesis of PE.

Endoglin (ENG) gene, which is expressed on syncytiotrophoblasts and transitioning cytotrophoblast cells of the placenta (St-Jacques, Forte, Lye, & Letarte, 1994), has been shown to participate in the regulation of placental trophoblast differentiation and invasion of the uterus during pregnancy (Caniggia, Taylor, Ritchie, Lye, & Letarte, 1997). In PE, shallow trophoblast invasion of the maternal spiral arteries restricts conversion of these arteries from small muscular vessels to large low-resistance vessels via the replacement of smooth muscle with fibrous tissue within the vessel wall. Without a sufficient physiologic conversion, limited lumen diameter and distensibility of the spiral arteries subsequently leads to the reduction in placental and fetal perfusion that is observed in PE (Brosens, Robertson, & Dixon, 1972; Zhou, Damsky, & Fisher, 1997). Conceptually, this process is referred to as Stage 1 in the two-stage model of PE (Roberts & Hubel, 2009). Therefore, an alteration in ENG function during placental implantation may contribute to PE pathogenesis.

ENG is also expressed on vascular endothelial cells (Gougos & Letarte, 1990) and is involved in the maintenance of vascular tone through the regulation of nitric oxide–dependent vasodilation (Jerkic et al., 2004; Toporsian et al., 2005). In PE, in addition to abnormal implantation, vascular endothelial function,

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including vasorelaxation and cell membrane permeability, is disrupted (American College of Obstetricians and Gynecologists [ACOG], 2002). Conceptually, this is referred to as Stage 2 in the two-stage model of PE and is associated with reduced organ perfusion, hypertension, proteinuria, and intravascular fluid loss (Roberts & Gammill, 2005; Roberts & Hubel, 2009). Therefore, an alteration in ENG function in the vasculature may contribute to PE pathogenesis.

Given the biologic plausibility of a role for ENG in PE pathogenesis, a plethora of research reports addressing ENG's involvement in the disorder has recently inundated the scientific literature. Despite the sizable amount of evidence being generated, which has primarily targeted ENG protein levels in PE, critical review and synthesis of the scientific literature addressing ENG messenger RNA (mRNA) expression in PE is lacking. We thus conducted this systematic review to critique/synthesize scientific literature that addresses the role of ENG expression in PE.

Data Collection Method

We used the PubMed and Ovid (MEDLINE) databases to identify articles addressing the role of ENG in PE from a human gene expression standpoint. The keywords we used were *preeclampsia, endoglin,* and *gene*. In PubMed, we combined all three keywords with the AND Boolean operator. Due to differences in MeSH terms across the databases, we used the following combination of keywords to retrieve articles in OVID (Medline): *endoglin* AND (*gene* OR *genes*) AND (*preeclampsia* OR *pre-eclampsia*). We limited the literature search, which covered literature through January 2011, to the English language and articles involving human research.

After completing the literature search, we reviewed abstracts of retrieved articles for relevance, excluding review articles, duplicate articles, and articles not addressing ENG expression in the context of PE. We also reviewed weekly PubMed e-mail updates related to endoglin and PE, along with reference lists of selected articles, to identify additional articles. After independent review, we met to discuss findings and synthesize results. We extracted from each study data related to study objective, design/approach, setting, subject information, phenotype, tissue type, data collection methods, statistics, and results and summarized them in tabular format.

Results

We selected 9 of the14 articles we identified from the initial literature search for inclusion. Of the excluded articles, 1 was a review article, 1 addressed ENG expression at the DNA level in PE, and 3 addressed ENG expression outside the context of PE. We also selected for inclusion of six additional articles that we identified from reference lists or PubMed e-mail updates. Table 1 includes a summary of the results and characteristics of the articles comparing ENG expression in human subjects with PE to a control group/groups. Table 2 includes a summary of the results and characteristics of the articles utilizing gene expression methods to investigate mechanisms that may explain the role of ENG in PE.

Discussion

Establishing a Role for ENG in PE via Gene Expression Studies

We conducted this systematic review in order to critique and synthesize scientific literature that addresses the role of ENG in PE from a human gene expression standpoint. In reviewing the 11 studies that compared gene expression levels of ENG between women with and without PE, we consistently found ENG expression to be significantly elevated in women with PE regardless of definition of PE, ancestral background, methods for gene expression analysis, tissue type, or time of specimen collection (Table 1). Four of these studies (Purwosunu et al., 2008; Purwosunu, Sekizawa, Okazaki, et al., 2009; Purwosunu, Sekizawa, Yoshimura, et al., 2009; Sekizawa et al., 2010), however, may represent one parent study instead of independent replicates. Although the number of independent studies would be reduced to eight, the support for ENG's involvement in PE remains strong.

Moreover, ENG expression was elevated throughout all three trimesters of pregnancy in women who developed PE, suggesting that ENG's role in PE is initiated early in pregnancy and sustained through delivery. Both first- and third-trimester placental samples of women who developed PE had significantly elevated levels of ENG expression (Farina et al., 2008; Nishizawa et al., 2007; Sitras et al., 2009; Toft et al., 2008; Venkatesha et al., 2006). Furthermore, the cellular and cellular-free (plasma) components of blood in women who developed PE had significantly elevated levels of ENG expression near the end of the first trimester and in the second and third trimesters (Farina et al., 2010; Purwosunu et al., 2008; Purwosunu, Sekizawa, Okazaki, et al., 2009; Purwosunu, Sekizawa, Yoshimura, et al., 2009; Sekizawa et al., 2010).

Further research is needed to understand why expression levels are altered and how these alterations are involved in the development of PE. Two approaches that may provide insight into why ENG expression is altered in PE are evaluating ENG at the molecular level (DNA) and exploring it from an epigenetic point of view. In the only study like it to date, Srinivas, Morrison, Andrela, and Elovitz (2010) examined the association between PE and allelic variation in an angiogenic pathway among Black ($N = 184$ cases and $N = 305$ controls) and White subjects ($N = 32$ cases and $N = 85$ controls) separately. Using the previously developed ITMAT-Broad-CARe, version 2 (IBCv2) array, they evaluated 124 tagging single nucleotide polymorphisms (SNPs) across the six candidate genes (vascular endothelial growth factor A, B, and C; fms-like tyrosine kinase 1 and 4; endoglin). Investigators failed to demonstrate a significant association between variation in ENG and PE; however, it is unclear if they fully evaluated the entire ENG. Further research examining the association between PE and allelic variation across the entire ENG in larger samples is needed.

Table 1. Cross-Sectional Studies Comparing Endoglin (ENG) Gene Expression in Subjects With Preeclampsia (PE) Versus Control Group/Groups

Citation (Population)	Design	Expression Evaluation	Phenotype	Tissue	Endoglin Results
Farina et al., 2008 (Italian)	Prospective case–control Matching (1:5): GA and fetal sex $N = 30$ (5 cases and 25 controls)	Candidate gene analysis via qRT-PCR	Case: PE; severe PE Control: Pregnant women without PE	Placenta: CVS at 11 weeks' gestation	ENG mRNA levels significantly higher in 11-week CVS in cases ($p < .001$)
Farina et al., 2010 (Italian)	Case–control Matching (1:8): GA and fetal sex $N = 99$ (11 cases and 88 controls)	Candidate gene analysis via qRT-PCR	Case: PE; severe PE Control: Pregnant women without PE	Blood: Cellular component at 10–14 weeks' gestation	ENG expression significantly higher in cellular component of blood in cases ($p < .001$)
Nishizawa et al., 2007 (Japanese)	Case–control + within-case subanalysis Matching: Maternal age, GA, and prepregnancy BMI *N* (microarray analysis): 10 cases and 4 controls	Nonparametric whole-genome analysis via microarray with qRT-PCR validation	Case: Severe PE Control: Normotensive subjects	Placenta: C-section prior to labor onset	ENG expression significantly upregulated in cases ($p = .000275$) ENG not differentially expressed between early-onset (<31 weeks) and late-onset PE placentas at delivery
Purwosunu et al., 2008 (Indonesian)	Case–control $N = 84$ (43 cases and 41 controls)	Candidate gene analysis via qRT-PCR	Case: PE; severe PE; HELLP Control: Healthy pregnant women	Blood: Cellular-free (plasma) component at 35–41 weeks' gestation	ENG expression significantly higher in cases ($p < .001$)
Purwosunu, Sekizawa, Okazaki, et al., 2009 (Indonesian)	Prospective cohort + subsequent case–control analysis Matching (1:5): GA at sample collection, maternal weight, and fetal gender $N = 372$ (62 cases and 310 controls)	Candidate gene analysis via qRT-PCR	Case: PE; severe PE; HELLP Control: Healthy pregnant women	Blood: Cellular-free component at 15–20 weeks' gestation	ENG expression significantly higher in plasma component of blood in cases ($p < .001$)
Purwosunu, Sekizawa, Yoshimura, et al., 2009 (Indonesian)	Case–control $N = 48$ (24 cases and 24 controls)	Candidate gene analysis via qRT-PCR	Case: PE; severe PE; HELLP Control: Healthy pregnant women	Blood: Cellular component at 35–41 weeks' gestation	ENG expression significantly higher in the cellular component of blood in cases ($p < .001$) In PE, ENG expression in cellular component of blood significantly correlated with SBP ($p < .001$, $R^2 = .631$) and proteinuria ($p < .001$, $R^2 = .671$)
Sekizawa et al., 2010 (Indonesian)	Prospective cohort + subsequent case–control analysis Matching (1:5): GA at sample collection, maternal weight, and fetal gender $N = 372$ (62 cases and 310 controls)	Candidate gene analysis via qRT-PCR	Case: PE; severe PE; HELLP Control: Healthy pregnant women	Blood: Cellular component at 15–20 weeks' gestation	ENG expression significantly higher in cellular component of blood in cases (statistics not provided)

(continued)

Table 1 (continued)

Citation (Population)	Design	Expression Evaluation	Phenotype	Tissue	Endoglin Results
Sitras et al., 2009 (Norwegian)	Case–control + within-case subanalysis Matching (1:1): Parity $N = 71$ (21 cases and 50 controls; analysis conducted on 16 cases and 21 random controls)	Nonparametric whole-genome analysis via microarray with qRT-PCR validation	Case: Severe PE; HELLP Control: Healthy women with uncomplicated pregnancies	Placenta: Delivery (C-section before labor and vaginal delivery)	ENG expression significantly upregulated (fourfold; $p \leq .01$) in cases ENG not a differentially expressed gene between early-onset (<34 weeks) and late-onset PE at delivery
Toft et al., 2008 (Norwegian)	Three-group comparative Matching: GA at delivery $N = 28$ (10 PE; 8 SGA; and 10 PE + SGA)	Nonparametric whole genome analysis via microarray Candidate gene analysis via qRT-PCR	Group 1: PE Group 2: SGA Group 3: PE + SGA	Placenta: C-section prior to labor onset	Microarray: No significant placental gene expression differences between study groups after correction for multiple comparisons ($p > .05$) Real-time qRT-PCR: ENG placental expression significantly higher in the PE + SGA group compared to PE and SGA groups ($p = .033$)
Tsai et al., 2011 (American)	Case–control $N = 60$ (23 cases and 37 controls)	Nonparametric whole-genome analysis via microarray with qRT-PCR validation	Case: PE Control: Pregnant women without PE	Placenta: Within 1 hr of delivery	ENG expression significantly upregulated in cases ($p = 1.5054 \times 10^{-08}$)
Venkatesha et al., 2006 (American)	Case–control Matching: GA N (30 normal-term; 8 normal-preterm; 11 mild PE; 17 severe PE without HELLP; and 11 severe PE with HELLP	Nonparametric whole-genome analysis via microarray with Northern blot confirmation	Case: PE; severe PE; HELLP Control: Healthy pregnant women	Placenta: Immediately following delivery	ENG expression upregulated (fourfold ↑) in cases (no p value reported)

Note. BMI = body mass index; C-section = cesarean section; CVS = chorionic villous sampling; DBP = diastolic blood pressure; GA = gestational age; HELLP = hemolysis, elevated liver enzymes, low platelets; mRNA = messenger RNA; qRT-PCR = quantitative reverse transcriptase–polymerase chain reaction; SBP = systolic blood pressure; SGA = small for gestational age.

Table 2. Studies Investigating Mechanisms That May Explain the Role of Endoglin Gene (ENG) in Preeclampsia

Citation	Design	Expression Evaluation	Tissue	Endoglin Results
Fujita et al., 2010	Mechanistic/experimental	Candidate gene analysis via qRT-PCR	BeWo choriocarcinoma cell line	ENG mRNA levels significantly ↑ in BeWo cells incubated under hypoxia compared to normoxia at 4 and 6 hr Treatment with kinase inhibitors for AKT and ERK attenuated hypoxia-induced ENG mRNA expression HIF-1α silencing significantly ↓ hypoxia-induced ENG mRNA expression
Henry-Berger et al., 2008	Mechanistic/experimental	Candidate gene analysis via Northern blot	Human choriocarcinoma JAR cell line	JAR cells treated with a synthetic liver X receptor agonist led to significant ↑ in ENG mRNA levels
Munaut et al., 2008	Mechanistic/experimental	Candidate gene analysis via qRT-PCR	First-trimester placental villous extracts ($N = 30$; 8–14 weeks' gestation)	ENG mRNA levels not modulated under hypoxia compared to normoxia after 48 hr
Rigourd et al., 2008	Mechanistic/experimental	Nonparametric whole genome analysis via microarray with qRT-PCR validation	JEG-3 choriocarcinoma cell line	Overexpression of STOX1 in JEG-3 choriocarcinoma cell line resulted in 2.23 fold induction of ENG expression

Note. qRT-PCR = quantitative reverse transcriptase-polymerase chain reaction; mRNA = messenger RNA.

Mechanisms Explaining the Role of ENG via Gene Expression Studies

Using gene expression as a tool, research has identified potential mechanisms that may help to explain ENG's role in PE (Table 2). In the reviewed studies investigating mechanisms that may explain ENG's role in PE, investigators identified the liver X receptor and the STOX1 transcription factor as potential regulators of ENG expression. Given that liver X receptors and ENG have been shown to be involved in placental implantation (Caniggia et al., 1997; Pavan et al., 2004), it is possible that the abnormal implantation observed in PE could be attributed to altered regulation of ENG by liver X receptor. STOX1 may also be involved in PE despite inconsistent results (Rigourd et al., 2008). Given that ENG expression is elevated in PE (Table 1) and that overexpression of STOX1 in a choriocarcinoma cell line (Table 2) leads to the induction of ENG expression (Rigourd et al.), it is plausible that STOX1 is involved in the development of PE and may epistatically contribute to ENG's role in PE.

The two remaining studies investigated the effect of hypoxia on ENG expression. Although one group of investigators did not find hypoxia to modulate ENG expression in first-trimester placental villous explants after 48 hr of incubation (Munaut et al., 2008), another group found hypoxia to significantly increase ENG expression in BeWo cells (choriocarcinoma cell line) after 4 and 6 hr of incubation (Fujita et al., 2010). These disparate results may be due to the different types of cells used to assess hypoxia's impact on ENG. Furthermore, results by Fujita et al. (2010) suggest that 3-kinase-AKT-MTOR-HIF-1α and ERK-HIF-1α signaling pathways influence ENG expression under hypoxic conditions.

Limitations of Studies Comparing ENG Expression in Subjects With PE Versus Control Group/Groups

Despite consistent findings, we did note limitations across studies and within individual studies that may impact the validity and overall interpretation of the gene expression results. One limitation that we found in all 11 studies is that they were cross sectional in nature. Although the studies demonstrated that ENG expression levels were elevated in all three trimesters of pregnancy cross-sectionally, studies utilizing a prospective, longitudinal approach have the ability to observe changes in gene expression across pregnancy in the same subjects. Ultimately, such information could provide further insight into ENG's role in PE throughout pregnancy. However, one must consider that, although the longitudinal assessment of gene expression from the blood is feasible, longitudinal assessment of placental gene expression is neither feasible nor ethical (e.g., second-trimester biopsies of pregnant women).

The variability in PE phenotype along with the variability in inclusion/exclusion criteria utilized to classify cases and controls among the reviewed studies represents another limitation that impacts the ability to compare results across studies. Such a limitation further hampers the ability to combine studies for the purpose of conducting a meta-analysis, which can be employed to estimate effect sizes.

Other noted limitations across studies were related to methods used to evaluate gene expression. Authors frequently failed to report the following steps involved in gene expression analysis: (a) performance of RNA quality/quantity control checks on extracted RNA prior to gene expression analysis, (b) use of an RNA stabilizer to prevent RNA degradation in the tissue until extraction and gene expression analysis, and (c) use of an endogenous control when conducting real-time quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR). Although these limitations may reflect editing to meet journal page constraints, they may also represent methodological flaws that could cause the validity of the findings to be in question.

Like the limitations noted across studies, limitations unique to several individual studies may also impact validity and generalization of results. In the study by Sitras et al. (2009), we noted two limitations that have the potential to impact placental gene expression. First, gestational age at delivery (sample collection) was significantly earlier in cases compared to controls. Tsai et al. (2011) also noted this limitation; however, more in-depth analysis by this group indicated that estimated gestational age had minimal independent contribution to their gene expression data. Ultimately, the magnitude of observed differences in ENG mRNA expression of the placenta may have been impacted by differences related to developmental stage of pregnancy. Although this issue may be mitigated through the matching of healthy controls to cases for gestational age at delivery, one must consider if a control who delivers preterm truly represents a "healthy" control. Second, the study sample included subjects who delivered via cesarean section prior to labor onset and those who delivered vaginally. Because placental gene expression profiles may differ between laboring and nonlaboring women, differences related to labor may influence study results. Such limitations deserve consideration when researchers are designing studies that evaluate relationships between gene expression and disease/health outcomes.

In the study by Toft et al. (2008), the absence of a normotensive control group and the use of a small sample size to conduct targeted gene analysis via qRT-PCR represent potential limitations. If the investigators had included a normotensive control group, it would be possible to compare differences in ENG expression between those with PE and those with an uncomplicated pregnancy within the study and across similar studies. As for the issue related to small sample size, significant results could ultimately indicate false-positive findings as opposed to large effect sizes.

Limitations of Studies Investigating Mechanisms That May Explain the Role of ENG in PE

We also noted several limitations in the studies investigating mechanisms that may explain the role of ENG in PE. In the study by Munaut et al. (2008), hypoxia did not modulate ENG expression in first-trimester placental villi culture explants. However, unlike the other candidate genes under study, authors

did not report the effects of hypoxia on ENG expression in human umbilical vein endothelial cells and immortalized first-trimester extravillous trophoblast. It is unclear if the authors simply omitted these results from the report or if they did not study the effects of hypoxia on ENG expression in these cell types. Because ENG is expressed on trophoblast and vascular endothelial cells, study of its expression in these cell types would have provided additional insight into hypoxia's effect on ENG's role in PE.

The use of cell lines to conduct research concerning biological processes in humans represents an additional limitation of the mechanistic studies reviewed. Although the choriocarcinoma cell lines utilized by Fujita et al. (2010), Henry-Berger et al. (2008), and Rigourd et al. (2008) were human in origin, the representativeness of an immortal cell line as "normal" decreases and the risk of genetic abnormalities increases with each cell passage. As a result, study findings may not accurately represent biological activities that are occurring in vivo.

Conclusion

PE represents a multisystem, hypertensive disorder of pregnancy that significantly contributes to maternal and fetal/neonatal morbidity and mortality worldwide. At present, the etiology of PE remains unknown, but gene expression studies included in this systematic review support ENG's involvement in the development of PE. Despite the methodological limitations in these studies, ENG expression was consistently elevated in women with PE. In addition, these studies showed that ENG's role in PE may be explained by several mechanisms that may represent a variety of biological functions.

Investigators can utilize the findings of this review to design future studies examining ENG's role in PE. First, research addressing methodological limitations found in the gene expression studies is needed to validate previous findings. Steps to mitigate such limitations include conducting and reporting RNA quality/quantity control checks, using RNA stabilizers to optimize RNA integrity of samples, using and reporting endogenous controls when appropriate, and collecting tissues of interest at comparable times (e.g., similar gestational age) between groups. Second, research that examines why ENG expression levels are altered and how these alterations are involved in PE development is needed. Ultimately, such studies have the potential to increase overall understanding of PE and to solve PE's etiologic puzzle, which may include ENG as one of its pieces.

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