2011

Racial Differences in the Genetics of Preeclampsia

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RACIAL DIFFERENCES IN THE GENETICS OF PREECLAMPSIA

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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July, 2011

Acknowledgement

I would like to take this opportunity to recognize some of the individuals who have guided and assisted me throughout my graduate education. I would like to express my sincere gratitude to my advisor, Dr. Jerome Strauss, for his mentorship and patience. I appreciate all of the time and effort he has given to ensure that I received a well rounded graduate experience. I am fortunate to have been able to train under such a positive physician-scientist role model. His dedication to family and a balanced life, while maintaining an incredibly productive career, have truly been an inspiration.

I would also like to thank the members of my graduate committee: Drs. Timothy York, Lindon Eaves, Scott Walsh, Phillip Gerk, and Joyce Lloyd. Their willingness to give time, advice, and guidance has been a tremendous help during my tenure as a graduate student. I would especially like to thank Dr. York for all of the time he has invested in helping me with the statistical aspects of my dissertation. He patiently deciphered my never-ending questions and guided me towards the answers. I would also like to thank Dr. Gordon Archer for giving me the opportunity to train in the physician-scientist program at VCU.

I would like to express my sincere appreciation to our collaborators, Dr. Roberto Romero, Dr. Juan Pedro Kusanovic, Dr. Michal Elotvitz, Dr. Sindhu Srinivas, and their labs for providing DNA from phenotyped subjects and for providing feedback on the manuscripts that have resulted from this work. I would also like to thank DaShaunda Hilliard for aiding me in the genotyping of the U.S. Black DNA samples. Additionally, I would like to acknowledge my funding sources for providing support for my training and research: (1) 1T32HLO94290-01A1 training grant from the National Heart, Lung, and Blood Institute, NIH, DHHS and (2) P60MD002256 from the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS.

A special thanks goes to my family and friends for their unending love and support. My parents, Denny and Nancy Hill, and my sister, Teresa Purkiss, have always stood behind me and encouraged me through all of life's ups and downs. I am also blessed by an incredibly large and supportive extended family. I would like to thank my grandparents, aunts, uncles, cousins, and friends for always having faith in me. I would especially like to thank my Uncle Chris for fostering my curiosity in science and nature from an early age and my Aunt Janet for being a true friend when I needed one the most. I would also like to thank Felix, for standing by my side and giving me confidence and strength when I most needed them.

Finally, I would like to recognize my grandmother, Mary Hobson, who died from preeclampsia in 1962. She has been an inspiration in my work and has made this dissertation a very personal and rewarding journey. I hope to honor her memory by helping to cure the disorder that took her life so early.

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List of Abbreviations

-2LL	**negative two times log likelihood**
2-ME	**2-methoxyestradiol**
A	**adenine**
ACOG	**American College of Obstetricians and Gynecologists**
AIC	**Akaike Information Criterion**
BMI	**body mass index**
C	**cytosine**
c^2	**shared environment variance component**
CI	**confidence interval**
COMT	**catechol-O-methyltrasferase**
COT	**children of twins**
CYP17A1	**cytochrome P450, family 17, *subfamily A,* polypeptide 1**
D'	**D prime between two loci**
eNOS	**endothelial nitric oxide**
e^2	**unique environment variance component**
ERAP2	**endoplasmic reticulum aminopepetidase**
f^2	**fetal genetic variance component**
G	**guanine**
h	**term for the difference in the contribution of shared environment between full and half-siblings**

HELLP	**hemolysis, elevated liver enzymes, and low platelets**
HIF-1α	**hypoxia inducible factor 1 alpha**
HLA-G	**human leukocyte antigen G**
IFNγ	**interferon gamma**
IL-1	**interleukin 1**
IL-6	**interleukin 6**
IL-8	**interleukin 8**
IL-10	**interleukin 10**
IL-12	**interleukin 12**
IL-16	**interleukin 16**
k	**number of free parameters**
LD	**linkage disequilibrium**
m^2	**maternal genetic variance component**
Met	**methionine**
MHC	**major histocompatibility complex**
MTHFR	**methylenetetrahydrofolate reductase**
n	**number**
NO	**nitric oxide**
OR	**odds ratio**
P	**p-value**

PCR	**polymerase chain reaction**
PE	**preeclampsia**
PlGF	**placental-derived growth factor**
R^2	**r-squared correlation coefficient between two loci**
RAAS	**rennin-angiotensin-aldosterone system**
RAS	**rennin-angiotensisn system**
ROS	**reactive oxygen species**
SAM	**S-adenosylmethionine**
S.E.	**standard error**
sENG	**soluble endoglin**
sFlt1	**soluble fms-like tyrosine kinase**
SNP	**single nucleotide polymorphism**
T	**thymine**
TGF-β	**transforming growth factor beta**
Th1	**T Helper Cell Type 1**
TNFα	**tumor necrosis factor alpha**
Val	**valine**
VEGF	**vascular endothelial growth factor**

Abstract

RACIAL DIFFERENCES IN THE GENETICS OF PREECLAMPSIA

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2011.

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Preeclampsia (PE), characterized by hypertension and proteinuria after 20 weeks of gestation, affects 5-8% of pregnancies worldwide. Although preeclampsia is a significant cause of maternal and perinatal mortality and morbidity, its etiology remains to be elucidated. Racial differences have been observed for preeclampsia, with U.S. Blacks having higher rates and more severe disease, compared to U.S. Whites and Hispanics. One potential source of racial differences in preeclampsia is genetic variation between populations. Genetic susceptibility to preeclampsia is well established, but the specific contributions of maternal vs. fetal genes, and how these vary among racial groups is poorly understood. This dissertation addressed racial differences in the genetics of preeclampsia in Chileans, U.S. Blacks, and U.S. Whites through candidate gene studies and variance components modeling. First,

we determined whether three genes, which are relevant to the pathophysiology of preeclampsia, *Catechol-O-methyltransferase (COMT), Methylenetetrahydrofolate reductase (MTHFR),* and *Endoplasmic reticulum aminopeptidase 2 (ERAP2),* were associated with the risk for preeclampsia in Chilean and U.S. Black mothers and fetuses. We found that the maternal *COMT* and an interaction between the fetal *COMT* and *MTHFR* were associated with the risk for preeclampsia in Chileans. We also found that the fetal *ERAP2* was associated with the risk for preeclampsia in U.S. Blacks. We next used structural equation modeling of a unique Children of Twins (COT), supplemented with full and half-siblings, study design to investigate the fetal genetic, maternal genetic, shared environmental, and unique environmental contributions to preeclampsia in U.S. Whites and Blacks. Through this modeling we uncovered a unique source of racial differences in preeclampsia. We found that U.S. Whites and Blacks showed a similar prevalence of preeclampsia in first births, but across the next three births, the prevalence in Whites declined to a greater degree than in Blacks. In conclusion we have identified specific maternal and fetal genes that contribute to the risk for preeclampsia. Furthermore, we have identified sources of racial differences in preeclampsia, which include differences in associations between *COMT, MTHFR, and ERAP2* and the risk for preeclampsia among populations and differences in the prevalence of preeclampsia across subsequent births between U.S. Whites and U.S. Blacks.

Chapter 1: General Introduction

Clinical overview of preeclampsia

Preeclampsia is one of the most common disorders of pregnancy, affecting 3-5% of pregnant women worldwide (1). In addition to being a common disorder of pregnancy, preeclampsia is a leading cause of maternal and perinatal morbidity and mortality. It is estimated that preeclampsia results in more than 60,000 maternal deaths worldwide (2), and accounts for 20% of maternal deaths in the United States (U.S.) each year (3). In the U.S. the overall case-fatality rate for preeclampsia is approximately 6.4 per 10,000 cases at delivery (3).

Preeclampsia is defined clinically as the presence of new-onset high blood pressure (defined as systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg) and proteinuria (300mg or greater in a 24 hr urine specimen) after 20 weeks gestation, or during the first 48 hours following delivery (1, 4). Preeclampsia can be further categorized into mild, severe (systolic blood pressure ≥ 160 mm Hg, diastolic blood pressure ≥ 110 mm Hg, or ≥ 5g protein in a 24 hr urine specimen), or the rare severe variant of HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets) (1, 4).

Serious health risks for both the mother and fetus/neonate are associated with preeclampsia. The mother is at risk for seizures (eclampsia), renal failure, pulmonary edema, stroke and death, whereas the fetus is at risk for intrauterine growth restriction,

death, and prematurity with attendant complications (5). A greater appreciation for long term complications of preeclampsia is also emerging. Preeclampsia is associated with earlier development of cardiovascular disease in both the mother and infant (6, 7). Furthermore, women who have had preeclampsia are at an overall greater risk of developing hypertension, cardiovascular disease, cerebrovascular disease, and kidney damage (8-11). Although preeclampsia is common and results in potentially devastating outcomes for both mother and child, the only definitive treatment is delivery. Inducing premature delivery does “cure” preeclampsia, however, it also places the neonate at risk of the sequelae of prematurity and low birth weight (5, 12).

In addition to limited treatment options for preeclampsia, there are few reliable methods to predict which women will develop preeclampsia. Several pregnancy-specific risk factors have been associated with a higher risk for preeclampsia. Nulliparous women or multiparous women with a new partner are at increased risk for preeclampsia (13, 14). Women with a history of preeclampsia, especially early onset disease, very young women, and women of advanced maternal age are also at increased risk for preeclampsia (4, 14, 15). Pregnancies with increased placental mass, including multi-fetal gestation and hydatidiform mole, are at higher risk of being complicated by preeclampsia (14, 16, 17). Finally, smoking decreases the risk for preeclampsia in some populations (18). Preexisting conditions in the mother also place her at higher risk of developing preeclampsia during pregnancy. These conditions include chronic hypertension, obesity, diabetes mellitus, renal disease, metabolic syndrome, and lupus (4, 14, 15). All of these risk factors are non-specific and, since

they are common among women who do not develop preeclampsia, provide little predictive power.

One of the main limitations to the prevention and treatment of preeclampsia is the inadequacy of the diagnostic criteria. Preeclampsia is diagnosed based on the presence of two basic clinical findings, which fail to capture the complexity of this syndrome. Preeclampsia is a heterogeneous disorder that encompasses a range of both classical and non-classical presentations based on the current definition. The classic presentation of preeclampsia includes a wide range in time of onset (20 weeks gestation to 48 hours post-partum), amount of proteinuria (300 mg/day to nephrotic syndrome), severity of disease, and course of progression (1, 4, 19, 20). There are also non-classical presentations that include the absence of proteinuria, absence of hypertension, HELLP syndrome, and eclampsia without previous signs and symptoms of preeclampsia (19, 20). Moreover, the diagnostic criteria for preeclampsia do not reflect the current understanding of the role of the placenta in this disease.

It is well established that preeclampsia is a disease of the placenta that results in the maternal phenotype of hypertension and proteinuria. The disease, therefore, is believed to begin early in the first trimester of pregnancy, yet maternal symptoms are usually not recognized until much later, with most presenting during the third trimester. The reliance on end stage signs and symptoms for diagnosis, and the diagnosis of preeclampsia late in the course of pregnancy reflect the inadequacies of the current criteria for diagnosis. A better understanding of the etiology of preeclampsia is needed so that a more adequate and comprehensive set of diagnostic criteria can be developed for the disorder.

Etiology of Preeclampsia

Despite much research, the proximate cause of preeclampsia remains poorly understood. Preeclampsia is believed to be a complex disorder influenced by multiple genetic, immunological, environmental, and social factors (21, 22). As a disorder of pregnancy, the pathophysiology of preeclampsia is also complex because it involves the interplay of two individuals, mother and fetus. As such there are both fetal and maternal contributions to this condition.

Preeclampsia is a placental disorder that results in the maternal phenotype of hypertension and proteinuria. The central role of the placenta is evidenced by the fact that delivery of the placenta, not the fetus, “cures” the disorder, and the fact that preeclampsia can develop with hydatidiform moles, where only placental tissue and no fetus is present. Consequently, abnormalities leading to preeclampsia are thought to occur early in pregnancy, and originate in fetal (placental) tissues, and the later stages of disease manifest in maternal tissues. This multistage process is outlined in Figure 1.1, which provides a general overview of our current understanding of the pathophysiology of preeclampsia (23). A discussion of the specific mechanisms thought to be important to the development of the disease is presented below.

Placentation is a critical event in the establishment of pregnancy, providing the vascular connection between the mother and fetus. During normal placentation cytotrophoblasts invade the uterine spiral arteries and transform them from small, high resistance vessels, to larger, low resistance vessels. This transformation ensures adequate placental profusion to maintain the fetus. Placental ischemia and hypoxia are central features of preeclampsia. Placentas from preeclamptic pregnancies show

shallow trophoblast invasion (24-26) and poor spiral artery remodeling (27-29). This is thought to result from an imbalance in angiogenic and anti-angiogenic factors, inflammatory factors, and hypoxia response factors (30-32). The angiogenic/ anti-angiogenic balance is tightly controlled by oxygen levels and normally ensures adequate remodeling of the spiral arteries (33). In preeclampsia abnormal concentrations of circulating angiogenic and anti-angiogenic factors have been widely reported. In particular, abnormalities in soluble fms-like tyrosine kinase (sFlt1), placental-derived growth factor (PlGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and soluble endoglin (sENG) have been described by a number of authors (34-41).

The next stage of preeclampsia shifts to a balance between the feto-placenta and the maternal system. Inflammation is also a key regulator of placentation and pro-inflammatory cytokines have been linked to poor placentation (31, 32, 42-44). Many of these cytokines, including interleukin 16 (IL-16), interleukin 10 (IL-10), tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), and interleukin 12 (IL-12), show altered levels in preeclamptic pregnancies (44). Normal pregnancy is considered to be a pro-inflammatory condition, but in preeclampsia, there is an exaggerated state of systemic inflammation (42). Oxidative stress in the placenta causes it to produce reactive oxygen species (ROS) that activate circulating leukocytes. In women with preeclampsia, there is an increase in neutrophil activation and transendothelial migration (45, 46). Activated neutrophils release a number of molecules which lead to increased inflammation, vascular oxidative stress, and an imbalance in vasoactive factors (46, 47). The placental release of pro-

inflammatory factors, or the pre-existence of increased inflammation in the maternal vasculature, could both contribute to the development of preeclampsia.

Additionally, pregnancy is a condition that requires immune tolerance of the fetus by the mother since fifty percent of the fetal genome is not shared with the mother. The maternal immune system in preeclampsia shows a different response profile that includes a predominant T Helper Cell Type 1 (Th1) immune response, which correlates to poor placentation, inflammation, and endothelial dysfunction (48). Furthermore, preeclampsia is associated with decreased levels of HLA-G, which normally protects the fetus from immune attack by the mother. Decreased levels of HLA-G have been reported in the circulation of women with preeclampsia, and reduced cell-surface expression has been reported in trophoblasts (49-52).

In this stage, systemic endothelial damage is the focus of disease. In preeclampsia, endothelial dysfunction results in vasospasm, increased vascular peripheral resistance, and vascular leakiness. The key vasodilator of the endothelium and regulator of peripheral vascular resistance in pregnancy is nitric oxide (NO). Women with preeclampsia showed impaired production of NO metabolites (53). Furthermore, a rat model showed that pharmacologic inhibition of NO production resulted in a preeclampsia-like phenotype that included hypertension, proteinuria, intrauterine growth restriction, and renal endothelial lesions (54, 55). NO is an important downstream mediator of many of the angiogenic and anti-angiogenic factors that show abnormalities in the circulation of women with preeclampsia (i.e. VEGF, TGF-β, sFlt1, and sENG) (53). NO is formed by endothelial nitric oxide synthase (eNOS) using L-arginine as substrate. Abnormalities in the production of NO and the activity of eNOS

have been documented in preeclampsia (56), and supplementation of women at high risk for preeclampsia with L-arginine significantly reduces risk of preeclampsia (57). In sum, NO is a likely end stage target of dysregulation in preeclampsia, and a major contributor to the systemic endothelial dysfunction that characterizes this disorder.

It is postulated that placental ischemia and hypoxia cause the placenta to release vasoactive and inflammatory factors that lead to intravascular inflammation (30-32), and endothelial dysfunction (35, 36, 58-60). Hypoxia Inducible Factor 1 alpha (HIF-1α) is a transcription factor that mediates cellular responses to hypoxia and its expression is altered in preeclampsia (33, 61, 62). HIF-1α expression is normally suppressed during pregnancy, but its up-regulation in preeclampsia leads to the expression of numerous genes which encode angiogenic, anti-angiogenic, and inflammatory molecules including those that encode proteins that are increased in the circulation of women with preeclampsia (i.e. sFlt1 and sENG). Moreover, the inflammatory response feeds into a positive feedback loop with hypoxia and endothelial damage and potentially intensifies these processes.

The reactions of the endothelium to damage, and to the attempt by the placenta to increase perfusion, are important to the progression of preeclampsia. A complicated cascade of events is set into motion by the maternal and fetal systems. The attempts to increase perfusion, correct the endothelial damage, and the toxic molecules released as a result of damage, lead to a worsening of the maternal and fetal conditions. Oxidative stress, leading to free radical production, vasoactive molecules, placental debris, cytokines, and additional inflammatory molecules continue to escalate the systemic endothelial damage. Major regulatory systems participate in the attempt to regulate the

increasing blood pressure and problems in these might contribute to the inability of the body to effectively regulate blood pressure. Both the maternal and fetal renin-angiotensin-aldosterone system (RAAS) show defects in preeclampsia (63, 64). The RAAS system is one of the primary means the body uses for regulating blood pressure.

It is also important to consider the pre-existing state of the maternal endothelium as a contributing factor to preeclampsia. The fragility of the endothelium determines the amount of insult it can incur before reaching the amount of damage necessary for preeclampsia to develop. Pre-existing conditions that put a mother at increased risk for developing preeclampsia generally include conditions that affect the endothelium and, therefore, put it at a weakened state prior to pregnancy. Pre-existing hypertension diminishes the mother's ability to effectively manage the additional damage to the vascular system. In these cases, a lower amount of damage can be tolerated before preeclampsia develops as compared to a healthy maternal system prior to pregnancy.

The final stage of preeclampsia is systemic maternal endothelial damage that results in the clinical phenotype of hypertension and proteinuria. Anti-angiogenic factors, systemic inflammation, immunologic factors, and hypoxia all play a part in creating the systemic damage (23). The endothelial damage can affect all areas of body, including the liver, kidneys, and brain (23). Endothelial damage can further progress to end organ effects that result in the numerous complications associated with preeclampsia.

Racial differences in preeclampsia

Racial differences in preeclampsia are well established. Compared to White women in the United States (U.S.), higher rates of preeclampsia have been found among U.S. Black women and lower rates have been found among U.S. Asian women

(65-68). Hispanic women have also been reported to have lower rates of preeclampsia compared to Black women (67, 68). Besides maternal race being associated with differences in preeclampsia rates, maternal-paternal racial discordance was reported to be associated with an increased incidence (66).

In addition to being at increased risk for developing preeclampsia, U.S. Black women often have more severe disease and are at a greater risk for severe complications (65). In the case of severe preeclampsia, U.S. Black women have more severe hypertension and need more antihypertensive medications than U.S. White women (69). Furthermore, U.S. Black women have higher case-fatality rates with reports showing that, compared to U.S. White women, U.S. Black women are three times more likely to die from preeclampsia (3, 70).

Attempts to identify the source of racial disparity in preeclampsia have, thus far, been unsuccessful. Studies have been unable to account for racial disparity by controlling for socioeconomic factors that are typically associated with differences in health between these groups (67, 68). A 10 year longitudinal study from 1993 to 2002 in New York also showed that despite greater access to care over time, the disparity between Whites and Blacks increased (68). There are several possible sources of racial differences that might be contributing to the disparity. For instance, differences in genetic causes of preeclampsia, differences in previously unmeasured environmental factors, or differences in the balance between genetic and environmental factors may exist between groups.

Currently, the majority of research on preeclampsia has relied on studies of White women to characterize the epidemiology and pathophysiology of preeclampsia. It

will be important to include different racial populations in preeclampsia research so that the cause(s) of racial disparities can be determined and incorporated into the development of treatment and management strategies.

Genetic contribution to preeclampsia

A familial predisposition to preeclampsia has been consistently demonstrated in studies from the U.S., Scotland, Iceland, Scandinavia, and Australia. These studies have shown an increased risk for first-degree relatives of women and a relatively strong heritability which is estimated to be 0.54 (95% CI, 0-0.71) (71-76). With evidence for a significant genetic contribution, research next focused on determining the maternal and fetal genetic effects. Studies have reported both a maternal and a fetal genetic component to preeclampsia (75, 77-79). The most substantial study of the respective maternal and fetal contributions to preeclampsia estimated the maternal effect to be 0.35 (95% CI, 0.33-0.36) and the fetal effect to be 0.20 (95% CI, 0.11-0.24), with maternally and paternally inherited genes assumed to act equally through fetal genetic effects (78). These estimates were standardized for a total variance of 1.0. The overall conclusion of research investigating a genetic predisposition to preeclampsia is that more than 50% of liability can be attributed to inheritance and that both maternal and fetal genes contribute.

Despite the wide acceptance of these estimates, there are serious limitations in the studies from which they were derived. The majority of evidence to support the heritability of preeclampsia comes from epidemiologic studies, which show familial aggregation patterns, and although these study designs strongly support a genetic component to preeclampsia, they are unable to estimate the contribution of manifest

genetic and environmental variables (71-79). The two main studies that have been cited for these estimates are both large Swedish twin studies. The 0.54 estimate of heritability from the largest published twin study was not statistically significant and had very wide confidence intervals (76). Furthermore, the study that attempted to separate maternal and fetal genetic estimates only included three unique family relationships, yet they estimated five parameters (78). This leaves the models unidentifiable and, in particular, leaves the fetal genetic estimate indistinguishable from maternal genetics and shared environment. These results leave questions about the actual values of these parameters, and warrant better study designs that can more confidently estimate the parameters. Specifically, maternal genetic, fetal genetic, shared environmental, and unique environmental parameters remain to be determined.

Although the relative importance of maternal and fetal genetic versus environmental contributions to preeclampsia has not been firmly established, linkage and candidate gene studies have shown a variety of chromosomal regions and genes to be associated with preeclampsia (80-82). Genes involved with endothelial dysfunction, oxidative stress, angiogenesis and thrombophilia have been associated with preeclampsia (80-82). The diversity of genes found to be associated with preeclampsia is reflective of the complex nature of this disorder and reaffirms many of the suspected mechanisms thought to contribute to disease. While numerous associations have been reported, they are overwhelmingly of maternal genes because genetic studies have focused on maternal genotypes. With the placenta playing a major role in the pathogenesis of preeclampsia, fetal genes should equally be considered and included in genetic studies.

Racial differences are also underappreciated in genetic research on preeclampsia. The studies on the heritability of preeclampsia and candidate gene studies have almost exclusively looked at White populations. With genetic differences between racial groups a potential source of racial disparities in preeclampsia, it is important to include different populations in genetic studies.

Overview of current research

The aim of this dissertation is to explore genetic contributions to preeclampsia through candidate gene studies and structural equation modeling techniques. This research provides new insights into the genetics of preeclampsia by including multiple racial groups in order to address issues of disparity, by separating between maternal and fetal genes, and by moving beyond a single variant approach in the candidate gene studies with the inclusion of haplotypes and gene-gene interactions.

The first three projects of this dissertation are candidate gene studies that determined whether three genes *Catechol-O-methyltransferase (COMT), Methylenetetrahydrofolate reductase (MTHFR), and Endoplasmic reticulum aminopeptidase 2 (ERAP2)*, were associated with the risk for preeclampsia. The functions of COMT, MTHFR, and ERAP2 are relevant to the pathophysiology of preeclampsia. Furthermore, animal models and/or human studies have suggested that genetic variation in these genes could contribute to preeclampsia. The predicted contributions of these genes, in reference to the stages of preeclampsia, are presented in figure 1.2. A detailed discussion on the predicted mechanisms can be found in Chapter 2 for *COMT* and *MTHFR,* and chapter 4 for *ERAP2.*

The first two projects specifically investigated the genetic contributions of *COMT* and *MTHFR* to preeclampsia. These projects included several unique features that improve upon current approaches typically employed in preeclampsia and pregnancy-specific research. First, the study populations for these projects were composed of Chilean maternal-fetal dyads and U.S. Black mothers and fetuses. By collecting samples from the mother and neonate, both maternal and fetal genes were analyzed, and more importantly, the combination of maternal and fetal genes in a single pregnancy was evaluated. This allowed for the discrimination between maternal and fetal genetic associations and for an analysis of both maternal and fetal effects in a single pregnancy. Second, the haplotype structure of *COMT*, composed of four single nucleotide polymorphisms (SNPs), was studied to account for more functionally relevant alleles of this gene. This provided more information on the gene and allowed for better characterization of *COMT* variants and their role in preeclampsia. Third, two genes were included in the study to investigate the potential for epistasis to contribute to the risk for preeclampsia. The inclusion of both genes in this study revealed new roles for the genes in preeclampsia and underscored the importance of considering how the combination of variants in networks of genes relates to disease risk.

The second candidate gene study of this dissertation investigated the association between the *ERAP2* gene and risk for preeclampsia. In addition to including both maternal and fetal genes in this study, again two racial populations were included to explore the problem of racial disparities in this disorder. The inclusion of Chileans and U.S. Blacks in this study revealed differences in the genetic factors that contribute to the risk for preeclampsia between racial groups.

The final project of this dissertation aimed to improve upon the current understanding of the heritability of preeclampsia by more precisely defining the contributions of genetic and environmental contributions to the development of preeclampsia. By using a unique children of twins study design that was supplemented with full sibling and half sibling relationships, this study was designed to discriminate between maternal genetic, fetal genetic, shared environmental, and unique environmental contributions to preeclampsia. Furthermore, both U.S. Whites and Blacks were studied, which provided unique insight into the source of racial disparity in preeclampsia.

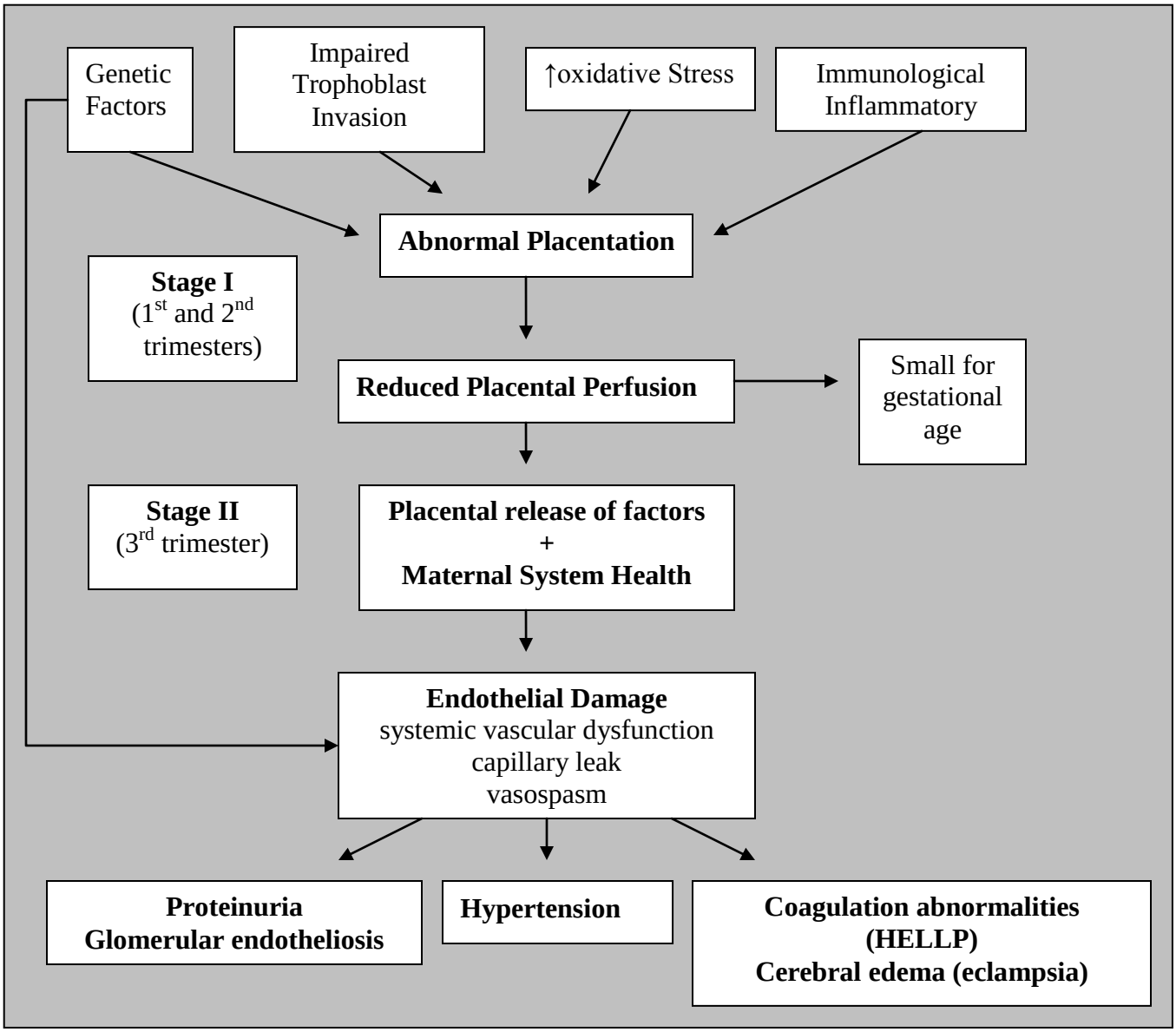

Figure 1.1. Summary of the pathophysiology of preeclampsia. In the early stage of disease, multiple factors contribute to placental dysfunction which leads to the placental release of anti-angiogenic factors and other inflammatory mediators. In the second stage of disease, the placental release of factors, in conjunction with maternal factors, leads to systemic endothelial damage. Systemic endothelial damage, ultimately results in hypertension, proteinuria, and other complications of preeclampsia. This figure has been adapted from Young *et al. (23)*.

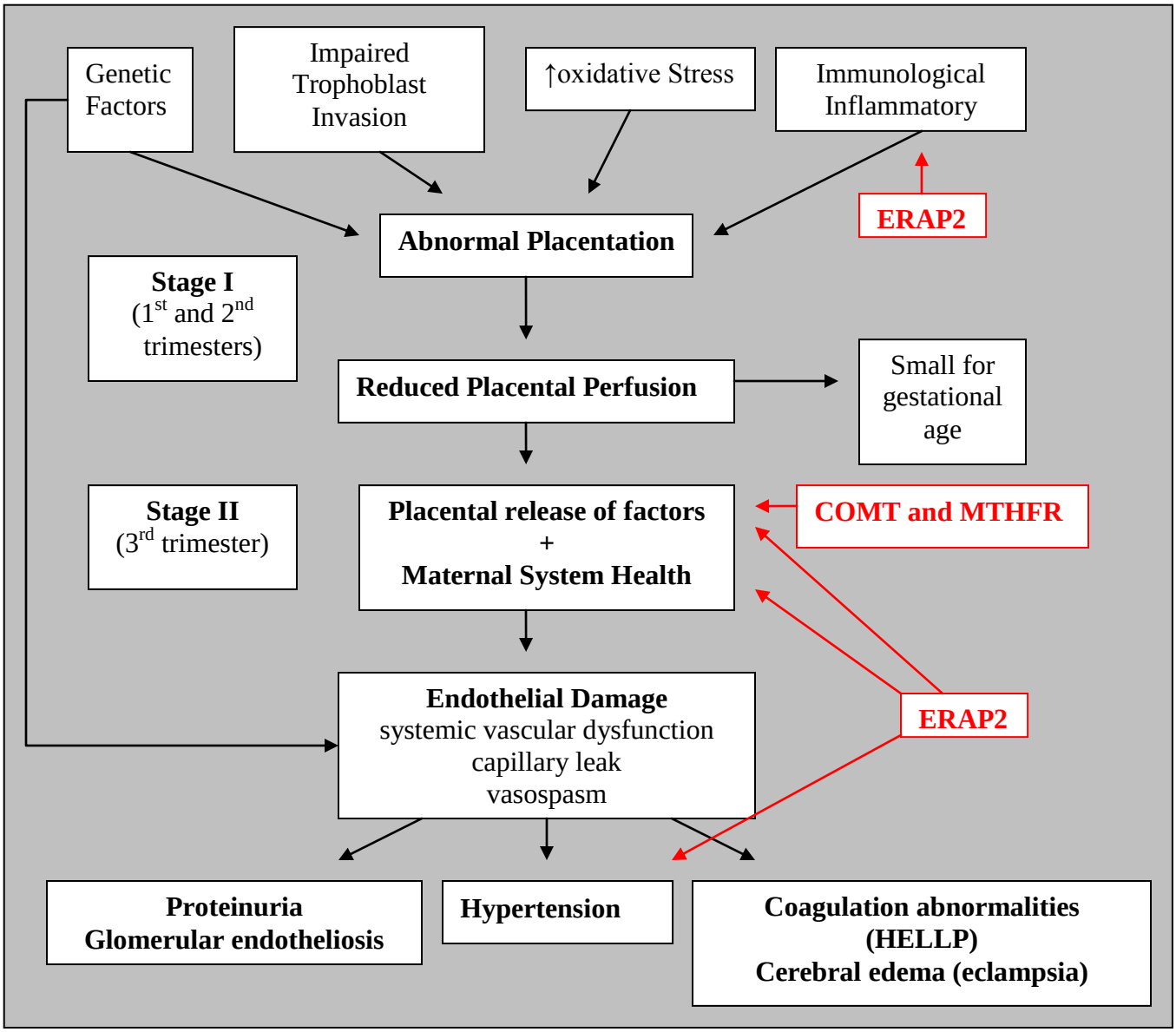

Figure 1.2. Summary of the pathophysiology of preeclampsia, including predicted involvement of Catechol-O-methyltransferase (COMT), Methylenetetrahydrofolate reductase (MTHFR), and Endoplasmic reticulum aminopeptidase 2 (ERAP2). We hypothesize that COMT and MTHFR are involved in placental hypoxia-driven disruption of angiogenic and anti-angiogenic factors. Specifically, that decreased COMT and MTHFR activity, leads to an up-regulation of Hypoxia Inducible Factor 1 alpha (HIF-1α). Increased HIF-1α is thought to lead to the inappropriate up-regulation of hypoxia-

induced genes and the placental release of factors that contribute to endothelial damage and the maternal phenotype. ERAP2 has the potential to contribute to preeclampsia in multiple ways. First, ERAP2 is involved in immunological and inflammatory processes. By altering these processes, ERAP2 could contribute to abnormal placentation. Second, ERAP2 is involved in inflammatory cytokine production and might contribute to the placental release of these factors. Third, the involvement of ERAP2 in inflammation could contribute to increased inflammation in the maternal system, and makes the maternal endothelium more sensitive to disease. Finally, ERAP2 is involved in blood pressure regulation and could contribute to hypertension in the later stage of disease. This figure has been adapted from Young *et al. (23)*.

Chapter 2: Epistasis between *COMT* and *MTHFR* in maternal-fetal dyads increases risk for preeclampsia

This chapter is published as:

Hill LD, York TP, Kusanovic JP, Gomez R, Eaves LJ, Romero R, Strauss JF 3rd. 2011 Epistasis between COMT and MTHFR in maternal-fetal dyads increases risk for preeclampsia. *PLoS One* 6(1):e16681

Abstract

One proposed mechanism of preeclampsia is placental hypoxia-driven imbalances in angiogenic and anti-angiogenic factors, causing endothelial cell dysfunction. C*atechol-O-methyltransferase (Comt)*-deficient pregnant mice have a preeclampsia phenotype that is reversed by exogenous 2-methoxyestradiol (2-ME), an estrogen metabolite generated by COMT. 2-ME inhibits Hypoxia Inducible Factor 1α, a transcription factor mediating hypoxic responses. COMT has been shown to interact with methylenetetrahydrofolate reductase (MTHFR), which modulates the availability of S-adenosylmethionine (SAM), a COMT cofactor. Variations in *MTHFR* have been associated with preeclampsia. By accounting for allelic variation in both genes; the role of *COMT* has been clarified. *COMT* allelic variation is linked to enzyme activity and four single nucleotide polymorphisms of this gene (SNPs) (rs6269, rs4633, rs4680, and

rs4818) form haplotypes that characterize COMT activity. We tested for association between *COMT* haplotypes and the *MTHFR* 677 C→T polymorphism and preeclampsia risk in 1103 Chilean maternal-fetal dyads. The maternal ACCG *COMT* haplotype was associated with reduced risk for preeclampsia (P = 0.004), and that risk increased linearly from low to high activity haplotypes (P = 0.003). In fetal samples, we found that the fetal ATCA *COMT* haplotype and the fetal *MTHFR* minor "T" allele interact to increase preeclampsia risk (p = 0.022). We found a higher than expected number of patients with preeclampsia with both the fetal risk alleles alone (P = 0.052) and the fetal risk alleles in combination with a maternal balancing allele (P < 0.001). This non-random distribution was not observed in controls (P = 0.341 and P = 0.219, respectively). Our findings demonstrate a role for both maternal and fetal *COMT* in preeclampsia and highlight the importance of including allelic variation in *MTHFR.*

Introduction

Preeclampsia (PE) affects 5-8% of pregnancies worldwide and is characterized by hypertension and proteinuria after 20 weeks of gestation (1). Although preeclampsia remains a significant source of maternal and perinatal mortality and morbidity, its etiology remains unclear. A genetic susceptibility to preeclampsia has been well established and genes involved with endothelial dysfunction, oxidative stress, angiogenesis and thrombophilia have been associated with preeclampsia (71, 80, 81, 83).

It has long been recognized that preeclampsia is a placental disorder that results in the maternal syndrome. Placental hypoxia is a key feature of this condition and

placentas from patients with preeclampsia show shallow trophoblast invasion (24-26) and failure of vascular transformation of the spiral arteries (27-29). During normal placentation, oxygen levels tightly control the balance between angiogenic and anti-angiogenic factors to ensure adequate remodeling of the maternal spiral arteries and sufficient placental blood supply (34). It is postulated that a hypoxia-driven disruption of the angiogenic balance causes the placenta to release factors that lead to intravascular inflammation (30-32), endothelial dysfunction (35, 36, 58-60) and the maternal phenotype. Indeed, abnormal concentrations of circulating angiogenic and anti-angiogenic factors including soluble fms-like tyrosine kinase (sFlt1), placental growth factor (PlGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and soluble endoglin (sENG) have been well documented in preeclampsia (34-41). Although abnormalities in these factors have been consistently demonstrated, there is no discernable pattern that characterizes preeclampsia, suggesting that a defect in an upstream regulator may contribute to the pathophysiology of preeclampsia.

2-methoxyestradiol (2-ME) is a natural metabolite of estradiol and it is generated by catechol-O-methyltransferase (COMT) in the placenta. 2-ME is a compound with diverse biological activities including inhibition of Hypoxia Inducible Factor 1α (HIF-1α) (33, 61). HIF-1α is a transcription factor that mediates cellular responses to hypoxia and its expression is altered in preeclampsia (33, 61, 62). Cytotrophoblastic invasion has also recently been reported to be modulated by 2-ME during hypoxic conditions (84). In collaboration with Kanasaki *et al.,* we found that the *Comt*-deficient pregnant mouse exhibits a preeclampsia phenotype similar to that found in human preeclampsia,

including hypertension, proteinuria and vascular and placental lesion; and the mouse preeclampsia-like phenotype is reversed by administration of 2-ME (61). In this report, circulating concentrations of 2-ME and placental COMT activity were significantly reduced in women diagnosed with preeclampsia, raising the possibility that altered production of 2-ME may contribute to the pathophysiology of preeclampsia by altering the placental response to hypoxia (61). Moreover, severe preeclampsia and fetal growth restriction have been associated with reduced placental COMT activity (85, 86). HIF-1α is an upstream regulator of many of the factors implicated in the angiogenic balance and endothelial dysfunction (34, 62). By modulating HIF-1α activity, COMT represents a point at which this upstream regulator could be disrupted.

Human allelic variation in *COMT* has been associated with changes in enzyme activity levels (87, 88). COMT is one of several enzymes that degrades catecholamines and is involved in vascular and metabolic homeostasis, including dopamine, epinephrine, norepinephrine, and catechol estrogens. The COMT enzyme is involved in a wide variety of physiological processes such as prefrontal cortex function and lipid metabolism and has been implicated in diseases such as schizophrenia, pain sensitivity, Parkinson's disease, and cancer (88-92). Previous studies investigating the role of genetic variation in *COMT* have largely focused on the single nucleotide polymorphism (SNP) rs4680 Val/Met, which has been associated with a modest 4-fold difference in activity (87). However, a recent functional analysis of 4 SNPs, rs6269, rs4633, rs4818, and rs4680, demonstrated that enzymatic activity is more precisely determined by three haplotypes of these SNPs, which result in a 25-fold difference in enzyme activity (88).

Preeclampsia is thought to be multifactorial in origin with multiple genes, environmental, and social factors acting in conjunction to cause disease (21, 22, 93). Variations in the methylenetetrahydrofolate reductase (*MTHFR*) gene have been associated with elevated homocysteine, a risk factor for endothelial dysfunction, vascular disease, and preeclampsia (94-97). Some previous studies have shown allelic variations in *MTHFR* to be associated with preeclampsia, although others have failed to replicate these associations (81, 98, 99). MTHFR modulates the availability of methyl groups (97), which are the cosubstrate for COMT (87) and Roffman *et al.* recently showed that stratifying *COMT* genotypes by *MTHFR* genotype revealed a role of *COMT* in prefrontal cortex function (87, 97, 100).

In the present case-control study, we investigated the association between *COMT* haplotypes and preeclampsia in 1,103 Chilean maternal-fetal dyads. Haplotype frequencies were determined by genotyping 4 SNPs from the *COMT* gene: rs6269, rs4633, rs4818, and rs4680. Based on previous findings of haplotype-specific differences in enzymatic activity and protein levels, we evaluated the relationship of the functional variation linked to *COMT* haplotype and preeclampsia (88). Finally, we assessed whether the relationship between *COMT* and preeclampsia was influenced by *MTHFR*.

Methods

Ethics Statement: This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of the Virginia Commonwealth University School of Medicine (IRB # HM12520). All

patients provided written informed consent for the collection of samples and subsequent analysis.

Study design and population: A case-control study was initiated by searching our clinical database and bank of biological samples and included Hispanic women and their neonates in the following groups: 1) Cases – women with preeclampsia and their neonates (*n* = 528 dyads); and 2) Controls – women who delivered at term with a normal pregnancy outcome and their neonates (*n* = 575 dyads). Participants received obstetrical care at the Sótero del Río Hospital in Santiago, Chile (an affiliated of the Pontificia Catholic University of Santiago, Chile). Exclusion criteria included: (1) known major fetal anomaly or demise; (2) multi-fetal pregnancy; (3) serious maternal medical illness (renal insufficiency, congestive heart disease, etc.); (4) refusal to provide written informed consent; and (5) a clinical emergency, which prevented counseling of the patient about participating in the study, such as fetal distress or maternal hemorrhage. All women provided written informed consent before collection of the samples. The use of clinical data and collection and utilization of maternal and neonatal blood for research purposes was approved by the Institutional Review Boards of the Sótero del Río Hospital and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS. Racially, the Chilean population is estimated at nearly 95% white and mestizo (mixed white and Amerindian); 3% Amerindian; and 2% other. Mixtures between the conquering Spaniards, largely Andalusians and Basques, and the Mapuches (Araucanians) produced the principle Chilean racial type (2002 census). There is no reported evidence to support differences in disease prevalence amongst

Chileans and there is no evidence to support the presence of group structure within this population. Therefore, population stratification was determined to not be a source of potential bias in this study population.

Clinical definitions: Preeclampsia was defined based on the presence of gestational hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg) and proteinuria (≥300 mg in a 24-hour urine collection, two or more dipstick measurement of 1+, or one or more dipstick measurement ≥2+) according to ACOG (1) and the National High Blood Pressure Education Program (1, 101). Patients were considered to have a normal pregnancy outcome if they did not have any medical, obstetrical, or surgical complication, and delivered a term neonate (≥37 weeks) of appropriate birth weight for gestational age (102) without complications.

Sample collection: Maternal blood samples were obtained from the mother at the time of enrollment in the protocol, and from the umbilical cord immediately after delivery before the detachment of the placenta. Samples were collected with a vacutainer into tubes containing EDTA. The plasma tubes were balanced and centrifuged at 1300*g* for 10 minutes at 4°C to separate cellular components from clear plasma, and the samples were stored at -70°C until assayed.

DNA extraction: DNA was extracted from maternal and cord blood with a Qiagen Autopure using standard procedures (Qiagen).

Genotyping: Single-nucleotide polymorphism analysis was performed using real-time allelic discrimination TaqMan assays (Applied Biosystems) with modifications. All PCR reactions contained 25-75 ng of DNA, 6.25 ul TaqMan Universal Master Mix (Applied Biosystems)(2x), 0.3 ul TaqMan Genotyping Assay (Applied Biosystems) (20x), and water for a final volume of 12.5 ul. Real-time PCR was performed on an ABI 7500 Fast Real-Time PCR Machine (Applied Biosystems) under the following conditions: 50^{o}C for 2 min, 95^{o}C for 10 min, and 40 cycles of amplification (92^{o}C for 15 sec and 60^{o}C for 1 min). For each cycle, the software determined the fluorescent signal from the VIC- or FAM- labeled probe (Applied Biosystems). Allelic discrimination for *COMT* was performed using TaqMan Genotyping assays C___2538746_1 for SNP rs6269, C___2538747_20 for SNP rs4633, C___2538750_10 for SNP rs4818, C__25746809_50 for SNP rs4680 (Applied Biosystems). Allelic discrimination for *MTHFR* was performed using TaqMan Genotyping assay C__1202883_20 for SNP rs1801133.

Statistical Analysis: Fisher's exact tests implemented in the PLINK software (103) were used to test individual SNPs for allelic associations with case-control status and to confirm Hardy-Weinberg equilibrium in the control group only. Inter-SNP linkage disequilibrium calculations for *COMT* were performed in Haploview (version 4.0) (104). Haplotype frequencies were also generated in PLINK based on the four *COMT* SNPs and both global and haplotype-specific tests were performed to test for frequency differences between disease status for maternal and fetal samples separately. Haplotypes with an independent effect were further investigated by multiple logistic

regression in R to condition by covariates known to influence rates of preeclamsia and to adjust for the correlation between maternal-fetal genotypes. These tests involved assigning haplotypes to subjects based on the most likely phase reconstructed haplotypes generated by the expectation-maximization algorithm implemented in PLINK. An additive term for the haplotype of interest was coded as 0, 1, or 2 based on copy number present. Based on the previously mentioned haplotype-specific functional information from Nackley *et al.* (88), we also coded *COMT* haplotypes to reflect enzymatic activity. *COMT* haplotypes were sequentially ordered 1 through 5 where 1 was ACCG/ACCG, 2 was ACCG/ATCA, 3 was ATCA/ATCA, 4 was ATCA/GCGG, and 5 was GCGG/GCGG. Interactive effects between the maternal ATCA *COMT* haplotype and maternal *MTHFR* and the fetal ATCA *COMT* haplotype and fetal *MTHFR* were tested using multiple logistic regression in R. The *MTHFR* was included as an additive term coded as 0, 1, or 2 based on copy number of the minor “T” allele. Permutation analysis in R with 10,000 iterations was used to compare models with and without significant interaction terms. Logistic regression in R was used to test for differences in clinical characteristics between disease classes for non-genetic variables.

Results

Table 2.1 displays the demographic and clinical characteristics of mothers and neonates from pregnancies with preeclampsia as well as controls. No significant differences were observed in maternal age or neonatal sex between groups. Consistent with previous epidemiologic studies, patients with preeclampsia showed a significantly higher body mass index (BMI, P <0.001) and fewer previous live births (P = 0.007). In

accordance with preeclampsia resulting in intrauterine growth restriction and indicated preterm birth, offspring born to women with preeclampsia showed a significantly lower gestational age at delivery and birthweight ($P < 0.001$).

Single SNP analysis revealed no associations between *COMT* polymorphisms rs6269, rs4633, rs4818, and rs4680 and preeclampsia in either maternal or fetal samples (Table 2.2). All SNPs were found to be in Hardy-Weinberg equilibrium in the maternal and fetal control samples separately. However, haplotype analysis showed the four SNPs to be in very high linkage disequilibrium (LD) for both maternal and fetal samples (Table 2.3). Three main haplotypes were identified: ACCG, ATCA, and GCGG (SNP order on the chromosome: rs6269, rs4633, rs4818, rs4680) and correspond to the low, intermediate, and high enzyme activities of COMT, respectively, identified by Nackley and colleagues (88).

The haplotype analysis of *COMT* frequency differences between cases and control subjects is shown in Table 2.4. A global test of differences among haplotypes reached statistical significance for maternal samples, but not for fetal samples ($P = 0.016$ and $P = 0.116$, respectively). Separate tests for haplotype-specific effects on disease class resulted in significant results for both the maternal ($P = 0.004$) and fetal ($P = 0.038$) ACCG haplotype (Table 2.4). This haplotype was observed more frequently in controls than cases for both maternal and fetal samples, indicating a possible protective effect. To control for the correlation of genotypes inherent in maternal-fetal dyads, we conditioned the maternal ACCG haplotype by the respective fetal ACCG haplotype. This resulted in only a significant effect of the maternal ACCG haplotype (maternal $P = 0.041$; fetal $P = 0.446$) on risk for disease and indicated that the effect of the ACCG

haplotype was maternally derived and initial significant result for the fetal ACCG haplotype was likely a result of the correlation of the fetal-maternal genotype.

Additional multiple logistic regression analysis was performed to include risk factors for preeclampsia (maternal age, BMI, and previous live births). Results of a final regression model, which only included covariates found to be significant in this population is shown in Table 2.5. Only the maternal ACCG haplotype (maternal P = 0.034, fetal P = 0.419) was observed to have a significant effect and was associated with a decreased risk of preeclampsia (OR = 0.796; 95% CI: 0.646, 0.982). Increased BMI was associated with an increased risk for preeclampsia (OR = 1.108; 95% CI: 1.076, 1.142) and a larger number of previous live births decreased the risk for preeclampsia (OR = 0.782; 95% CI: 0.695, 0.880).

Nackley et al. demonstrated in a mammalian expression system that *COMT* haplotypes resulted in an ordered progression of enzyme activity with the ACCG haplotype showing a 18-25 fold decrease in activity and the ATCA haplotype showing a 2.5-3 fold decrease in activity compared to the GCGG high activity haplotype (88). Results of a multiple logistic regression model that included maternal and fetal terms to reflect enzymatic activity of the *COMT* haplotypes (ie., each coded as an ordinal variable), maternal BMI, and previous live births are shown in Table 2.6. When maternal and fetal terms were analyzed separately, both show a significant positive relationship with increasing enzymatic activity and preeclampsia risk (P = 0.003 and P = 0.014 respectively). However, when both maternal and fetal terms were included in the same model, again the fetal association decreased in significance (P = 0.561) and the maternal ordered *COMT* haplotypes approached significance (P = 0.061).

Obesity is a major risk factor for preeclampsia and our results demonstrated BMI to be strongly associated with preeclampsia in this population (47). COMT metabolizes catecholamines, which are known to modulate lipid mobilization (105). Several studies have found modest associations between obesity and the rs4680 (Val^{158}Met) SNP of *COMT* (106, 107). The potential for COMT to contribute to preeclampsia risk through maternal BMI led us to investigate whether the association between maternal *COMT* haplotype and preeclampsia risk in our study could be explained by a relationship between *COMT* and BMI. PLINK was used to test for allelic associations between individual SNPs and BMI, where BMI was the quantitative phenotype. Haplotype frequencies were also generated in PLINK based on the four *COMT* SNPs and haplotype-specific tests were performed to test for frequency differences in association with BMI. Table 2.7 shows results for analyses that tested the relationship between maternal *COMT* and BMI in our study population. No significant associations between C*OMT* haplotypes or individual maternal SNPs and BMI were observed.

The potential for *MTHFR* to influence risk for preeclampsia both through a single gene effect and an interaction with *COMT* was studied (81, 98, 100). The rs4680 loci of *COMT* encodes for an amino substitution (Val^{158}Met) and COMT protein with methionine at position 158 is reported to be less stable and with reduced activity (87). However, this instability can be overcome by the binding of the methyl cosubstrate for COMT, s-adenosylmethionine (SAM) (108). *MTHFR* modulates the availability of methyl substrates for COMT, including SAM, and the minor “T” allele of the rs1801133 SNP of *MTHFR* has been associated with reduced MTHFR activity and reduced production of SAM (97). The ATCA haplotype of *COMT* is the only observed haplotype to have the

"A" allele at the rs4680 loci and we postulated that an interaction between the ATCA haplotype of *COMT* and the minor "T" allele of the *MTHFR* rs1801133 SNP would result in a further decrease of COMT activity because there would not be adequate levels of SAM to stabilize the COMT protein. We therefore tested for epistasis between the ATCA *COMT* haplotype and SNP rs1801133 of *MTHFR*. Results of a multiple logistic regression model that included maternal and fetal terms for the interaction between the ATCA *COMT* haplotype and *MTHFR* are shown in Table 2.8. A significant interaction (*P* = 0.022) between the fetal ATCA *COMT* haplotype and the fetal *MTHFR* was observed, which resulted in an increased risk for preeclampsia (OR = 1.370; 95% CI: 1.048, 1.792). The critical value for the test statistic associated with the interaction term was also estimated using permutation techniques and resulted in an empirical p-value of 0.023. No association was found between SNP rs1801133 in *MTHFR* and preeclampsia in either maternal or fetal samples (*P* = 0.470 and *P* = 0.225 respectively).

Our results revealed both a maternal protective effect and a fetal risk effect. Since our data included maternal-fetal dyads, we looked at the combination of maternal and fetal effects in a single pregnancy, focusing on the fetal high risk genotypes. Within cases we looked at the proportion of pregnancies that had two fetal high risk *COMT* ATCA x *MTHFR* "T" combinations with no maternal protective *COMT* ACCG allele and those that contained the two fetal high risk combinations with a balancing maternal *COMT* ACCG allele. We observed a higher than expected number of patients with preeclampsia with both the fetal risk alleles alone (Chi-square = 3.789; *P* = 0.052) and the fetal risk alleles in combination with a maternal balancing protective allele (Chi-

square = 22.549; $P < 0.001$). This non-random distribution across dyads was not observed in controls ($P = 0.341$ and $P = 0.219$, respectively).

Discussion

Preeclampsia is a common disorder of pregnancy with potentially devastating complications (1, 4). Placental hypoxia and endothelial cell dysfunction are central features of this disorder (34). One proposed mechanism for preeclampsia is placental hypoxia-driven imbalance of angiogenic and anti-angiogenic factors (34-40), resulting in endothelial dysfunction (35, 36, 58, 60, 109). 2-Hydroxyestrogens are metabolized by COMT to produce 2-ME, a compound with diverse biological activities including inhibition of HIF-1alpha, a transcription factor that mediates cellular response to hypoxia (33, 61, 62). Epidemiologic data has consistently demonstrated a strong genetic susceptibility to preeclampsia and *COMT* has been identified as a candidate gene for preeclampsia studies (61, 71, 76, 80). In the present study, we found that the maternal ACCG haplotype of *COMT*, which is associated with low enzyme activity, was associated with a significantly reduced risk for preeclampsia in this population, and that the risk increased in a linear fashion from low to high activity alleles. We also found that epistasis between fetal *COMT* and *MTHFR*, which is associated with decreased enzyme activity as well, was associated with significantly increased risk for preeclampsia in this population.

We have previously reported that a *Comt* -/- mouse model exhibits a preeclampsia phenotype that is reversed by administration of 2-ME (61). This model lead us to postulate that decreased production of 2-ME in humans, as a result of allelic

variation in *COMT,* contributed to the development of preeclampsia (61). The results of our current study showed that a maternal haplotype of *COMT,* which likely results in decreased levels of maternal 2-ME production, was in fact protective and decreased the risk for preeclampsia. In contrast, an interaction between a fetal haplotype of *COMT* and fetal *MTHFR,* which likely results in decreased level of fetal 2-ME production, increased the risk for preeclampsia as was initially predicted. A significant limitation to the *Comt* -/- mouse model is that COMT was absent in both the maternal and fetal compartments. By being deficient in both compartments, it is unclear whether the preeclampsia-like phenotype is a result of deficiencies in both compartments, or rather a deficiency in only one of the compartments. Although our results appear contradictory and do not support our initial hypothesis, we would like to propose that they are not inconsistent with the mouse model. We speculate that decreased maternal COMT activity would be beneficial by increasing the production of 2-ME by the placenta and that a placental loss of COMT activity is the key deficiency that contributes to the development of preeclampsia.

Previous research on genetic variation in the *COMT* gene has largely focused on a single SNP rs4680, which causes a valine to methionine substitution at position 158 ($Val^{158}Met$) of the membrane bound version of the protein and position 108 of the soluble form. This amino acid substitution has been associated with a 4-fold decrease in activity in homozygote individuals (87). Three additional SNPs, rs6269, rs4633, and rs4818, have recently been reported by Nackley *et al.* to contribute to haplotype structures with rs4680 (88). Although only rs4680 encodes an amino acid change, the additional polymorphisms are predicted to cause changes in mRNA secondary structure

and thus, alter translation of the gene. Three main haplotypes were identified GCGG, ATCA, and ACCG and functional analysis in a mammalian expression system revealed changes in enzyme activity ranging from a decrease of 2.5-3 fold with the intermediate haplotype, ATCA, to a decrease of 18-25 fold with the low activity haplotype, ACCG (88). Decreased activity of the low ACCG haplotype was attributed to low translation of the protein, while the decreased activity of the ATCA haplotype was attributed to impaired stability of the protein as a result an amino acid substitution at SNP rs4680 (88). Our study supports this conclusion in that we found the four SNPs to be in very high linkage disequilibrium and we identified the same three haplotypes in our Chilean population. Our single SNP analysis showed no significant results, but haplotype analysis revealed a significant association between *COMT* and preeclampsia.

These results are in agreement with several recent studies that identified *COMT* haplotype associations with attention deficit hyperactivity disorder, pain sensitivity, and Parkinson's disease (110-112). Even more compelling, however, is our finding that preeclampsia risk changed in a linear fashion when we ordered haplotypes by reported enzymatic function. The ATCA haplotype was between the ACCG and GCGG haplotypes, with the ACCG haplotype being associated with the lowest risk for preeclampsia, and the GCGG haplotype with the highest risk. This progressive risk supports the assertion by Nackley et al. that ATCA represents the intermediate activity haplotype, while ACCG and GCGG are the extremes (88). The results reported herein have significant implications not only for research in preeclampsia, but also for future studies investigating genetic variation in the *COMT* gene. Our results suggest that investigating only the *COMT* rs4680 158Val → Met polymorphism provides incomplete

information because it fails to recognize haplotype structures, which account for larger variations in enzyme activity. *COMT* haplotypes therefore can provide clarification of the role of *COMT* alleles in disease. The identification of haplotypes which modulate enzyme activity to a greater degree than a single polymorphism might explain the sometimes contradictory results of previous genetic association studies with other common diseases (113, 114).

When considering the *COMT* gene independently, our results show that the maternal low activity haplotype of *COMT*, ACCG, was associated with a significantly lower risk for preeclampsia. The lower activity of the ACCG *COMT* haplotype has been reported to be the result of changes in mRNA secondary structure that lead to decreased translation of COMT protein (88). Thus, the protective maternal effect of *COMT* on the risk for preeclampsia is likely the result of a translational mechanism. This significant association between *COMT* and preeclampsia highlights the importance of this gene in preeclampsia, but does not support the causative mechanism suggested by the *Comt* knock out mouse. The finding of a protective effect of a low COMT activity haplotype may suggest that reduced catecholamine metabolism or 2-ME production in the maternal compartment spares the placenta from hypoxia. Decreased metabolism of catecholestrogens in the maternal compartment would increase the amount circulating through the placenta and increase the potential production of 2ME in this compartment.

Obesity is a major risk factor for preeclampsia and increased BMI was highly correlated with increased risk for preeclampsia in our study (47). COMT metabolizes catecholamines including dopamine, epinephrine, norepinephrine, and chatecholestrogens. Catecholamines modulate lipid mobilization by means of adipose

tissue lipolysis (105). Specifically, estrogen and androgen concentrations are involved in body fat regulation and estradiol appears to stimulate preadipocyte proliferation and differentiation. Additionally, 2-ME has been shown to inhibit preadipocyte proliferation and differentiation in vitro (105). In 2004, Tworoger *et al.* found that the rs4680 SNP in *COMT* modestly affected exercise-induced fat loss and in 2008, Annerbrink *et al.* found that the rs4680 SNP was associated with increased waist-to-hip ratio and abdominal sagittal diameter (106, 107). In our study, there was no association between maternal *COMT* haplotypes or individual SNPs and BMI (Table 5). While we can conclude that BMI was not driving the relationship between *COMT* and the risk for preeclampsia in the present study, it is not valid to extend these results past the current sample since individuals with preeclampsia are oversampled in a case-control study.

Our *COMT* x *MTHFR* interaction findings support a similar finding by Roffman *et al.* that the low COMT activity allele only in the presence of a low MTHFR activity allele was associated with prefrontal cortex function (100). In our study, the ATCA haplotype of *COMT* increased the risk for preeclampsia when the fetus also carried a low activity allele of the *MTHFR* gene, characterized by the minor “T” allele at SNP rs1801133. Unlike the translational mechanism proposed to govern the maternal *COMT* effect, the mechanism for the *COMT* x *MTHFR* interaction is most likely to be post-translational. The ATCA haplotype of *COMT* is the only haplotype that alters the amino acid sequence and it results in a thermodynamically unstable COMT protein. However, this instability can be overcome by the binding of its cosubstrate, S-adenosylmethionine (SAM) (108). MTHFR modulates the availability of SAM and the minor “T” allele at SNP rs1801133 results in lower production of SAM (97). Therefore, when the fetus carries

the “T” allele of *MTHFR* and the ATCA haplotype of *COMT*, the instability of COMT is not rectified and lower COMT activity is realized.

The identification of a fetal genetic risk factor for preeclampsia is an important step in understanding the cause(s) of preeclampsia. The placenta is fetal tissue and our results strengthen the argument that primary defects in the placenta play a central role in the development of preeclampsia. Additionally, our findings are consistent with the observations of reduced placental COMT activity and suggest that loss of activity in the fetal compartment of the *Comt* -/- mice appears to be responsible for the development of disease in this model (61).

Our findings have demonstrated both protective and risk alleles for *COMT* in association with the risk for preeclampsia. By investigating maternal-fetal dyads, we were able to explore the implications of both, seemingly contradictory, associations in a single pregnancy. We found a disproportionately high number of cases with two fetal ATCA *COMT* x *MTHFR* “T” risk combinations and with the two fetal risk combinations and one maternal ACCG protective *COMT* allele. What is most striking about our results however is the much larger chi-square value for the preeclamptic pregnancies that have two fetal risk combinations and one balancing maternal protective allele versus only having two fetal risk combinations and no balancing maternal allele (chi-square 22.549 vs. 3.789). The more significant nonrandom distribution of women with preeclampsia with a maternal protective ACCG *COMT* allele suggests that when the fetus is at high risk, it is preferred to have a maternal protective ACCG *COMT* allele to potentially offset the risk to some degree. Consequently, these pregnancies may be more viable than pregnancies where the fetus is at high risk, but has no maternal protection from

disease. This hypothesis might help explain the findings in the -/- *Comt* mouse model and the observation that women with preeclampsia have lower levels of circulating 2ME (61). The balancing combination of maternal and fetal *COMT* alleles results in low COMT activity in both the maternal and fetal compartments and this mimics the low COMT profile of the knockout mouse. Although our results appear consistent with the mouse model, further studies are needed to understand how COMT behaves differently in the maternal and fetal compartments to modulate the risk for preeclampsia.

Racial differences in preeclampsia have been identified. Increased rates of preeclampsia have been found in U.S. Black, Hispanic, and Asian women compared to white women, with U.S. Blacks having the highest rates (66). Additionally, maternal-paternal racial discordance has been associated with an increased incidence (66). These findings indicate that differences in genetic causes of preeclampsia may exist between racial groups. Global variation in allele frequencies for both *COMT* and *MTHFR* have also been demonstrated (97, 115-120). Moreover, allele frequencies for both genes are known to not only differ among major racial categories such as European, Asian, and U.S. Black, but substantial variation has also been demonstrated in subpopulations of each (97, 115-120). Racial variation in each gene raises the possibility that different alleles of *COMT* and *MTHFR* could contribute to preeclampsia risk in different racial groups. The Chilean population in this study has a genetic background most similar to Western Europeans, and in particular, those of Spanish descent (2002 census). Future studies among different racial populations are needed to determine if our results can be extended to other racial groups.

Table 2.1. Maternal and fetal characteristics of pregnancies diagnosed with preeclampsia and controls.

	Preeclampsia	Controls	*P*-value
Number of dyads	528	575	-
Maternal Age (*years)*	26.3 (7.5)	26.1 (6.2)	0.692
BMI (kg/m^2)	26.4 (5.4)	24.5 (4.4)	< 0.001
Previous live births	0.80 (1.19)	0.99 (1.08)	0.007
Birthweight (*grams)*	2805.7 (815.7)	3423.2 (303.0)	< 0.001
Gestational age at delivery (*weeks)*	36.8 (3.4)	39.7 (1.1)	< 0.001
Fetal sex (*% female)*	45.8	53.3	0.492

Data are presented as means (SD). BMI, body mass index.

Table 2.2. *COMT* single SNP analysis for maternal and fetal samples with and without preeclampsia.

COMT SNP	Frequency Preeclampsia	Frequency Controls	Chi-square	*P*-value
Maternal				
rs6269	0.316	0.284	2.676	0.104
rs4633	0.372	0.347	1.551	0.214
rs4818	0.307	0.274	2.897	0.091
rs4680	0.367	0.348	0.858	0.373
Fetal				
rs6269	0.308	0.287	1.142	0.305
rs4633	0.376	0.355	1.040	0.309
rs4818	0.299	0.283	0.705	0.425
rs4680	0.376	0.354	1.154	0.288

SNP, single nucleotide polymorphism.

Table 2.3. *COMT* pair-wise SNP linkage disequilibrium analysis for maternal and fetal samples.

COMT SNP Combination	D' (95% C.I.)	R^2
Maternal		
rs6269:rs4633	1.000 (0.98, 1.00)	0.239
rs6269:rs4818	0.991 (0.97, 1.00)	0.936
rs6269-rs4680	0.974 (0.93, 1.00)	0.225
rs4633:rs4818	0.987 (0.95, 1.00)	0.222
rs4633:rs4860	0.984 (0.97, 1.00)	0.959
rs4818:rs4680	0.980(0.94, 1.00)	0.217
Fetal		
rs6269:rs4633	1.000 (0.98, 1.00)	0.242
rs6269:rs4818	0.984 (0.96, 1.00)	0.940
rs6269-rs4680	0.994 (0.96, 1.00)	0.239
rs4633:rs4818	0.994 (0.96, 1.00)	0.232
rs4633:rs4860	0.992 (0.98, 1.00)	0.984
rs4818:rs4680	0.994 (0.96, 1.00)	0.232

SNP, single nucleotide polymorphism; D', D prime between the two loci; C.I., confidence interval; R^2, correlation coefficient between the two loci.

Table 2.4. *COMT* haplotype analysis for mothers and fetuses with and without preeclampsia.

Haplotype	Frequency Preeclampsia	Frequency Controls	Chi-square	DF	*P*-value
Maternal					
Global Test			8.260	2	0.016
ATCA	0.373	0.348	1.531	1	0.216
GCGG	0.310	0.277	2.807	1	0.094
ACCG	0.317	0.375	8.112	1	0.004
Fetal					
Global Test			4.308	2	0.116
ATCA	0.381	0.359	1.302	1	0.254
GCGG	0.302	0.283	0.907	1	0.341
ACCG	0.318	0.360	4.308	1	0.038

COMT haplotype SNP order: rs6269, rs4633, rs4818, rs4680. DF, degrees of freedom. Maternal and fetal samples were analyzed separately. The Global test of association indicated that, in maternal samples, a significant difference in allele frequencies between cases and controls existed amongst the *COMT* haplotypes. When haplotypes were tested individually, both the maternal and fetal ACCG *COMT* haplotypes were found more frequently in controls than cases.

Table 2.5. Logistic regression model of primary risk factors for preeclampsia including presence of the ACCG *COMT* haplotype.

Term	Estimate (S.E.)	*P*-value	Odds Ratio (95% C.I.)
Maternal ACCG	-0.228 (0.107)	0.034	0.796 (0.646, 0.982)
Fetal ACCG	-0.092 (0.114)	0.419	0.912 (0.729, 1.140)
Maternal BMI	0.103 (0.015)	< 0.001	1.108 (1.076, 1.142)
Previous live births	-0.246 (0.060)	< 0.001	0.782 (0.695, 0.880)
Intercept	-2.289 (0.378)	< 0.001	-

COMT haplotype SNP order: rs6269, rs4633, rs4818, rs4680. S.E., standard error; C.I., confidence interval; BMI, body mass index. When both maternal and fetal *ACCG* haplotypes from the maternal-fetal dyads were included in a single model, the maternal ACCG *COMT* remained significantly associated with reduced risk for preeclampsia. The fetal ACCG *COMT* haplotype is not associated with risk for preeclampsia after correcting for shared genetics between the mother and fetus.

Table 2.6. Logistic regression model of primary risk factors for preeclampsia including *COMT* haplotype specified according to reported enzymatic activity.

Term	Estimate (S.E.)	*P*-value	Odds Ratio (95% C.I.)
Maternal haplotypes *	0.166 (0.089)	0.061	1.180 (0.992, 1.406)
Fetal haplotypes †	0.052 (0.090)	0.561	1.053 (0.883, 1.257)
Maternal BMI	0.081 (0.019)	<0.001	1.084 (1.045, 1.126)
Previous live births	-0.236 (0.076)	0.002	0.790 (0.680, 0.917)
Intercept	-2.560 (0.529)	<0.001	

COMT haplotype SNP order: rs6269, rs4633, rs4818, rs4680. Ordered *COMT* haplotypes: 1=ACCG/ACCG, 2=ACCG/ATCA, 3= ATCA/ATCA, 4=ATCA/GCGG, 5=GCGG/GCGG. Haplotypes were ordered from 1 (low activity) to 5 (high activity) in accordance with reported information on enzyme activity[33]. Maternal haplotypes showed increased risk for preeclampsia as haplotypes moved from low to high activity alleles. * If maternal term fitted in model without fetal haplotypes $P = 0.003$, OR = 1.221 (1.073, 1.390). † If fetal term fitted in model without maternal haplotypes $P = 0.014$, OR = 1.179 (1.034, 1.345). S.E., standard error; C.I., confidence interval; BMI, body mass index.

Table 2.7. Maternal *COMT* analysis for body mass index.

COMT	Estimate (S.E.)	*P*-value
SNP		
rs6269	0.120 (0.231)	0.605
rs4633	-0.182 (0.222)	0.413
rs4818	0.092 (0.234)	0.693
rs4680	-0.192 (0.223)	0.390
Haplotype		
ATCA	-0.191*	0.392
GCGG	0.114*	0.628
ACCG	0.093*	0.675

Estimate is reported with (Standard Error) for SNPs. *COMT* haplotype SNP order: rs6269, rs4633, rs4818, rs4680. *Standard errors are not calculated for haplotypes by PLINK. S.E., standard error; SNP, single nucleotide polymorphism.

Table 2.8. Logistic regression model of *COMT-MTHFR* interaction risks for preeclampsia.

Term	Estimate (S.E.)	*P*-value	Odds Ratio (95% C.I.)
Maternal ACCG	-0.220 (0.126)	0.080	0.803 (0.627, 1.027)
Fetal ACCG	-0.126 (0.134)	0.345	0.882 (0.678, 1.146)
Maternal ATCA	0.017 (0.173)	0.921	1.017 (0.725, 1.428)
Fetal ATCA	-0.323 (0.174)	0.064	0.724 (0.515, 1.018)
Maternal MTHFR	-0.038 (0.143)	0.792	0.963 (0.727, 1.274)
Fetal MTHFR	-0.084 (0.145)	0.563	0.919 (0.692, 1.222)
Maternal ATCA : Maternal MTHFR	-0.028 (0.138)	0.840	0.972 (0.742, 1.274)
Fetal ATCA : Fetal MTHFR	0.315 (0.137)	0.022	1.370 (1.048, 1.792)
Maternal BMI	0.102 (0.015)	< 0.001	1.107 (1.075, 1.140)
Previous live births	-0.252 (0.060)	< 0.001	0.777 (0.691, 0.874)
Intercept	-2.082 (0.437)	< 0.001	-

COMT haplotype SNP order: rs6269, rs4633, rs4818, rs4680. *MTHFR* SNP rs1801133. S.E., standard error; C.I., confidence interval; BMI, body mass index. An interaction between the fetal ATCA *COMT* haplotype and the minor “T” allele of *MTHFR* significantly increased the risk for preeclampsia; after correcting for risk factors identified to modulate risk in this population.

Chapter 3: *COMT* and *MTHFR* variation and Preeclampsia in U.S. Blacks: Implications of ancestral differences in haplotype structure and minor allele frequency

Introduction

Racial differences exist for preeclampsia, with U.S. Blacks having a higher incidence and more severe disease than Whites (65-68). This racial disparity in disease burden places U.S. Black women and their babies at higher risk for severe complications and/or death (3, 70). Unfortunately, there are only a limited number of studies that have addressed the causes of the racial differences in preeclampsia among racial groups. Moreover, these studies were unable to account for the difference by adjusting for differences in socioeconomic indicators (67, 68). Thus, the source(s) of the observed racial disparities remains unknown. One potential factor that could contribute to racial differences in preeclampsia is genetic variation. Based on ancestral differences in genetic backgrounds, different genes or different variants of the same gene may be associated with preeclampsia in different populations. Heterogeneity in genetic associations with preeclampsia could result from differences in which genes contribute to disease, differences in polymorphisms within disease-causing genes, differences in linkage between genetic regions, or differences in allele frequencies among racial groups.

In Chapter 2, we demonstrated that *Catechol-O-methyltransferase (COMT)* and *Methylenetetrahydrofolate reductase (MTHFR)* were associated with the risk for preeclampsia in Chilean maternal-fetal dyads. In the current study, we extended the findings presented in Chapter 2 to include a U.S. Black population. By including U.S. Blacks in our studies of these genes, we can determine if our findings were specific to the Chilean population, or whether they are common between Chileans and Blacks.

Methods

U.S. Black women and fetuses from Pennsylvania and Michigan were included in this study. The study population consisted of 836 maternal (424 preeclamptic, 412 normal) and 837 fetal (375 preeclamptic, 462 normal) samples. Race was self-reported and U.S. Blacks were identified by selecting “Black, not Hispanic” on the self-report form. Of the 1673 total samples, 78% were paired maternal-fetal dyads. A full description of the study population has been previously described (121), and can be found in Chapter 4. Additionally the maternal and fetal characteristics of the population are presented in table 4.1. The methods and analysis applied to this population are the same as described for the Chilean population in Chapter 2. Power calculations were made using the Genetic Power Calculator (122), assuming a 5% disease prevalence.

Results

Table 3.1 shows minor allele and haplotype frequencies for *COMT* and *MTHFR* SNPs in U.S. Blacks. In comparison to Chileans, the minor “T” allele frequency of *MTHFR* for U.S. Blacks was significantly lower (~0.45 vs 0.11, respectively). The minor

allele frequencies (MAF) of the four *COMT* SNPs were also different in comparison to the Chileans (Table 2.2). The four *COMT* SNPs form 7 haplotypes in U.S. Blacks: ACCG, ATCA, GCGG, GCCG, GCCA, ACGG, and ATCG. This was a more variable haplotype structure than was seen in U.S. Whites or Chileans (88, 123). However, the three haplotypes observed in U.S. Whites and Chileans were among the most frequent haplotypes observed in U.S. Blacks.

The *COMT* gene alleles vary in frequency among major racial groups such as Europeans, Asians, and U.S. Blacks, and substantial variation is also observed in subpopulations of each broad racial group (97, 115-117). The racial variation in *COMT* haplotype frequency can be seen in Table 3.2, which provides a comparison of the most frequent haplotypes observed in U.S. Blacks, U.S. Whites, and Chileans. Notably, U.S. Whites show a lower frequency of the ACCG haplotype, U.S. Blacks show a lower frequency of the GCGG haplotype, and U.S. Blacks have a fourth haplotype (GCCG) that accounts for 20.3% of the observed haplotypes.

Consistent with our study of a Chilean population, no associations between *COMT* SNPs or the rs1801133 SNP of *MTHFR* and preeclampsia were found in U.S. Blacks (Table 3.3). However, in contrast to the Chilean study, associations between *COMT* haplotype and preeclampsia were not observed in either U.S. Black women or fetuses (Table 3.4). The lack of association remained even when the analysis was limited to a minor allele frequency of >0.05. The association between the maternal ACCG haplotype and preeclampsia detected in the study of Chileans was also not replicated.

Based on the results presented in Chapter 2, we tested for interactions between *COMT* haplotype and the rs1801133 SNP of *MTHFR* in the U.S. Black population. Because of the low minor allele frequency of the *MTHFR* SNP*,* a dominant model for this gene was used in the analysis. This was necessary to attain a sufficient number of observations in combination with individual *COMT* haplotypes to meet the criteria of the statistical test. Epistasis between the ATCA *COMT* haplotype and *MTHFR* was not observed in either U.S. Blacks mothers or fetuses in association with preeclampsia (Table 3.5). In addition to testing for epistasis between the ATCA haplotype and the *MTHFR* SNP*,* all other haplotypes were examined. No significant associations were observed between any other *COMT* haplotype and the *MTHFR* SNP and risk for preeclampsia. It is important to note that haplotypes with very low frequencies did not provide sufficient numbers of observations in combination with the *MTHFR* minor allele to satisfy the requirement of the statistical assumptions.

Discussion

In this study, we showed that Chileans, U.S. Whites, and U.S. Blacks have differences in allele and haplotype frequencies for both *COMT* and *MTHFR.* This suggests that ancestral differences in the genetic backgrounds of these three populations might contribute to heterogeneity in genetic contributions to preeclampsia. We showed that U.S. Blacks have a looser haplotype structure for *COMT,* which is consistent with a more diverse genetic background for this population. In contrast to the three haplotypes of *COMT* observed in Chileans and U.S. Whites, U.S. Blacks were observed to have seven haplotypes (88, 123). Global variation in allele frequency for

individual SNPs in *COMT* has been well established (115-117), and our study indicated that these differences are also seen at the haplotype level. Additionally, U.S. Blacks had a minor allele frequency for the rs1801133 SNP of *MTHFR* that was one-quarter of the minor allele frequency observed in Chileans. The difference in minor allele frequencies for the *MTHFR* SNP was consistent with published data on this SNP (115, 118, 119).

In Chileans, we demonstrated that the maternal ACCG haplotype of *COMT* decreased the risk for preeclampsia, and that the fetal ATCA haplotype of *COMT* in combination with the fetal minor “T” allele of rs1801133 in *MTHFR* increased the risk for preeclampsia. These findings did not replicate in the study of U.S. Black women and fetuses. There are two potential reasons for the differences in results for these two studies. First, based on the increased number of haplotypes, this study had lower power to detect a primary effect of *COMT* than the Chilean study. Additionally, the number of maternal and fetal samples was lower for this group, further decreasing power. Based on the haplotype frequency of the maternal ACCG in U.S. Blacks, our study only had 20% power to detect the effect size indicated by the Chilean study. With the increased number of haplotypes and the much lower minor allele frequency for the *MTHFR* SNP, our ability to detect a gene-gene interaction was also substantially diminished. This was due to the very low number of observations of each *COMT* haplotype in combination with the minor allele of the *MTHFR* SNP. A much larger sample size will be needed to achieve adequate power for these effects to be detected, if they are present. The second possibility for the lack of replication is that these genes do not contribute to preeclampsia in U.S. Blacks. Preeclampsia is a syndrome with a wide range of clinical presentations. Furthermore, racial differences in this disorder are reflective of the

heterogeneous nature of preeclampsia. Racial groups differ in their genetic backgrounds, raising the possibility that there are differences in genetic contributions to preeclampsia between populations. *COMT* and *MTHFR* may not contribute to preeclampsia in a significant way in U.S. Blacks, or different variants in the genes, which were not measured in our study, could be important for the development of the disorder in this population. The low minor allele frequency of the *MTHFR* SNP suggests a lower potential for this gene to contribute to preeclampsia in U.S. Blacks compared to Chileans or U.S. Whites.

Differences between populations in associations between *COMT* haplotypes and the risk for preeclampsia have also recently been seen. Roten *et al.* reported that the maternal ACCG haplotype of *COMT* was associated with an increased risk for recurrent preeclampsia (124). “Flip-flop” associations (opposite associations) are now recognized to reflect the complexity of common diseases, rather than contradictory findings (125). The “flip-flop” phenomenon was proposed by Lin *et al.* in 2007 and argues that opposite associations actually confirm an association, and can be explained by multilocus effects and variation in interlocus correlations. In particular, one of the examples explored by Lin *et al.* was contradictory associations that have been reported between *COMT* and schizophrenia. They showed that variable linkage disequilibrium (LD) could account for the reported differences (125). An alternative explanation for these findings could be that, based on shared genetics between mother and fetus, the observed association reflected a fetal genetic contribution. Without measuring the fetal genes, it is difficult to separate between maternal and fetal effects. Future studies that account for more complex contributions to the risk for preeclampsia by gene networks or physiologic

pathways that include genes, environmental, and social factors may clarify the similarities and differences between the genetics of preeclampsia in different racial populations.

Table 3.1. Genotype and Haplotype frequencies for *MTHFR* and *COMT* SNPs in U.S. Blacks.

Group	Gene	SNP/Haplotype	Minor Allele	Minor Allele Frequency
Maternal	*MTHFR*	rs1801133	T	0.107
	COMT	rs6269	G	0.378
		rs4633	T	0.315
		rs4818	G	0.197
		rs4680	A	0.296
		ACCG		0.271
		ATCA		0.271
		GCGG		0.160
		GCCG		0.193
		ATCG		0.043
		ACGG		0.036
		GCCA		0.025
Fetal	*MTHFR*	rs1801133	T	0.114
	COMT	rs6269	G	0.397
		rs4633	T	0.321
		rs4818	G	0.218
		rs4680	A	0.295
		ACCG		0.243
		ATCA		0.268
		GCGG		0.174
		GCCG		0.194
		ATCG		0.051
		ACGG		0.043
		GCCA		0.024

SNP, single nucleotide polymorphism. *COMT* single SNP frequencies are listed first, followed by *COMT* haplotypes formed by those SNPs. *COMT* haplotype SNP order: rs6269, rs4633, rs4818, rs4680.

Table 3.2. Comparison of maternal *COMT* haplotype frequencies across three distinct racial populations.

Haplotype	U.S. Blacks	U.S. Whites	Chileans
ACCG	0.278	0.103	0.375
ATCA	0.249	0.473	0.348
GCGG	0.163	0.375	0.277
GCCG	0.203	-	-

COMT haplotype SNP order: rs6269, rs4633, rs4818, rs4680. Haplotype frequencies were calculated from control subjects only. The U.S. White frequencies were taken from Nackley *et al.* (88) and the Chilean frequencies were taken from Hill *et al. (123)*. The GCCG haplotype was only observed in the U.S. Black population, in addition to 3 additional haplotypes (GCCA, ACGG, ATCG), which comprised the remaining 10.7% of haplotypes observed.

Table 3.3. *COMT* and *MTHFR* SNP analysis for U.S. Black mothers and fetuses with and without preeclampsia.

Gene	SNP	Frequency Preeclampsia	Frequency Controls	Chi-square	*P*-value
Maternal					
MTHFR	rs1801133	0.101	0.110	0.232	0.630
COMT	rs6269	0.354	0.391	1.580	0.209
	rs4633	0.351	0.298	3.472	0.062
	rs4818	0.184	0.203	0.661	0.416
	rs4680	0.328	0.280	3.094	0.079
Fetal					
MTHFR	rs1801133	0.105	0.122	1.102	0.294
COMT	rs6269	0.382	0.410	1.313	0.252
	rs4633	0.326	0.317	0.169	0.681
	rs4818	0.230	0.207	1.115	0.291
	rs4680	0.296	0.294	0.005	0.956

SNP, single nucleotide polymorphism.

Table 3.4. *COMT* haplotype analysis for U.S. Black mothers and fetuses with and without preeclampsia.

Haplotype	Frequency Preeclampsia	Frequency Controls	Chi-square	DF	*P*-value
Maternal					
Global Test			4.159	6	0.655
ACCG	0.267	0.273	0.057	1	0.811
ATCA	0.306	0.255	3.585	1	0.058
GCGG	0.152	0.165	0.369	1	0.544
GCCG	0.180	0.200	0.709	1	0.400
ATCG	0.043	0.043	<0.001	1	0.987
ACGG	0.030	0.038	0.602	1	0.438
GCCA	0.023	0.025	0.045	1	0.832
Fetal					
Global Test			4.399	6	0.623
ACCG	0.247	0.240	0.100	1	0.752
ATCA	0.273	0.266	0.078	1	0.780
GCGG	0.180	0.169	0.272	1	0.602
GCCG	0.176	0.049	3.107	1	0.078
ATCG	0.053	0.049	0.170	1	0.680
ACGG	0.050	0.038	1.210	1	0.271
GCCA	0.022	0.026	0.228	1	0.633

COMT haplotype SNP order: rs6269, rs4633, rs4818, rs4680. DF, degrees of freedom. Maternal and fetal samples were analyzed separately. The Global tests of association compared all haplotypes in a single analysis. The individual haplotype tests compared each haplotype to “all others” (i.e., a combined group of any haplotype not equivalent to the test haplotype). The global test and individual haplotype tests all indicated that no significant difference in allele frequencies between cases and controls existed amongst the *COMT* haplotypes.

Table 3.5. Logistic regression model of *COMT-MTHFR* interaction risks for preeclampsia in U.S. Black mothers and fetuses.

Group	Term	Estimate (S.E.)	*P*-value
Maternal	ATCA haplotype	0.250 (0.149)	0.093
	MTHFR SNP	-0.071 (0.278)	0.798
	ATCA : MTHFR	-0.049 (0.316)	0.876
	Intercept	-0.819 (0.129)	<0.001
Fetal	ATCA haplotype	0.097 (0.137)	0.475
	MTHFR SNP	0.074 (0.240)	0.756
	ATCA : MTHFR	-0.421 (0.300)	0.160
	Intercept	-0.132 (0.111)	0.236

COMT haplotype SNP order: rs6269, rs4633, rs4818, rs4680. MTHFR SNP rs1801133. S.E., standard error; C.I., confidence interval. Epistasis is indicated by a ":" between terms. Based on low minor allele frequency for *MTHFR* (~0.11), MTHFR was coded as a dominant term in the model in order to attain a sufficient number of *COMT* ATCA haplotype : *MTHFR* minor "T" allele of rs1801133 observations for analysis.

Chapter 4: Fetal *ERAP2* variation is associated with preeclampsia in U.S. Blacks

This chapter is published as:

Hill LD, Hilliard DD, York TP, Srinivas S, Kusanovic JP, Gomez R, Elovitz MA, Romero R, Strauss JF 3rd. 2011 Fetal *ERAP2* variation is associated with preeclampsia in African Americans in a case-control study. *BMC Medical Genet* 12:64.

Abstract

Background: Preeclampsia affects 3-8% of pregnancies and is a major cause of maternal and perinatal morbidity and mortality worldwide. This complex disorder is characterized by alterations in the immune and vascular systems and involves multiple organs. There is strong evidence for a genetic contribution to preeclampsia. Two different single nucleotide polymorphisms (SNPs) in the *endoplasmic reticulum aminopeptidase 2 (ERAP2)* gene were recently reported to be associated with increased risk for preeclampsia in two different populations. *ERAP2* is expressed in placental tissue and it is involved in immune responses, inflammation, and blood pressure regulation; making it is an attractive preeclampsia candidate gene.

Furthermore, *ERAP2* expression is altered in first trimester placentas of women destined to develop preeclampsia.

Methods: A case-control design was used to test for associations between two SNPs in *ERAP2*, rs2549782 and rs17408150, and preeclampsia status in 1103 Chilean maternal-fetal dyads and 1637 unpaired U.S. Black samples (836 maternal, 837 fetal).

Results: We found that the fetal minor allele (G) of rs2549782 was associated with an increased risk for preeclampsia in the U.S. Black population (P = 0.009), but not in the Chilean population. We found no association between rs17408150 and risk for preeclampsia in the Chilean population. Association between rs17408150 and risk for preeclampsia was not tested in the U.S. Black population due to the absence of the minor allele in this population.

Conclusions: We report an association between fetal *ERAP2* and preeclampsia in a U.S. Black population. In conjunction with previous studies, which have found maternal associations with this gene in an Australian/New Zealand population and a Norwegian population, *ERAP2* has now been associated with preeclampsia in three populations. This provides strong evidence that *ERAP2* plays a role in the development of preeclampsia.

Introduction

Preeclampsia (PE) affects 3-8% of pregnancies worldwide, with rates varying by race, and leads to potentially devastating complications for both the mother and fetus (1, 66). Preeclampsia is clinically characterized by high blood pressure and proteinuria, usually occurring after 20 weeks of gestation. Although this serious disorder is common during pregnancy, its etiology remains poorly understood (1). Preeclampsia is considered a disease of the placenta, with shallow trophoblast invasion (24-26) and poor spiral artery remodeling (27-29) being central features of this disorder. It is postulated that immune, vascular, and inflammatory disturbances participate in the placental dysfunction that ultimately produces the preeclampsia phenotype (126).

A genetic susceptibility to preeclampsia has been established with both maternal and fetal genes contributing to disease (66, 71, 75, 77-81, 83). Preeclampsia is a multi-factorial trait, with multiple genes, as well as environmental and social factors contributing to disease risk (21, 22, 93). Johnson *et al.* recently reported that *Endoplasmic reticulum aminopepetidase 2* (*ERAP2*) was associated with preeclampsia in an Australian/New Zealand family-based study and a Norwegian case-control study of maternal samples (127). Although *ERAP2* was associated with risk for preeclampsia in both populations, different polymorphisms of the gene were identified in each group. *ERAP2* is expressed in the syncytiotrophoblast and it is a member of the oxytocinase subfamily of M1 aminopeptidases, which are known to play a critical role in the maintenance of normal pregnancy (49, 128, 129). Additionally, ERAP2 is involved in the regulation of blood pressure, immune responses, and pro-inflammatory cytokine production (49, 50, 130-132). It was recently shown that *ERAP2* expression was altered

in first trimester placentas of pregnancies destined to develop preeclampsia (133). The involvement of *ERAP2* in multiple pathways known to influence the risk for preeclampsia, its expression in placental tissue, and the previously described altered expression of *ERAP2* in placentas before maternal symptoms developed (133); suggest that the fetal *ERAP2* gene contributes to the development of preeclampsia.

In the present study, we investigated whether the previously described associations between *ERAP2* and risk for preeclampsia (127) replicated in other racial groups and extended our study design past maternal only samples to also include fetal samples. We examined the association between *ERAP2* and risk for preeclampsia in two distinct case-control cohorts: Chilean (1103 maternal-fetal dyads) and U.S. Black (836 maternal and 837 fetal samples). We genotyped the two SNPs in *ERAP2*, rs17408150 and rs2549782, that were previously identified as being associated with preeclampsia. Our results demonstrate that the rs2549782 SNP of the fetal *ERAP2* gene is significantly associated with risk for preeclampsia in the U.S. Black population; further suggesting that this gene plays a key role in the development of disease and may provide insight into the disparity between preeclampsia rates between racial groups.

Methods

Chilean study design and population: A case-control study was initiated by searching the clinical database and bank of biological samples of the Perinatology Research Branch (*Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS) and included Hispanic women and their neonates in the

following groups: 1) Cases – women with preeclampsia and their neonates (*n* = 528 dyads); and 2) Controls – women who delivered at term with a normal pregnancy outcome and their neonates (*n* = 575 dyads). Participants received obstetrical care at the Sótero del Río Hospital in Santiago, Chile (an affiliate of the Pontificia Católica de Chile in Santiago, Chile). Exclusion criteria included: (1) known major fetal anomaly or demise; (2) multi-fetal pregnancy; (3) serious maternal medical illness (renal insufficiency, congestive heart disease, etc.); (4) refusal to provide written informed consent; and (5) a clinical emergency, which prevented counseling of the patient about participating in the study, such as fetal distress or maternal hemorrhage. All women provided written informed consent before collection of the samples. The use of clinical data and collection and utilization of maternal and neonatal blood for research purposes was approved by the Institutional Review Boards of the Sótero del Río Hospital, the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS and Virginia Commonwealth University. Racially, the Chilean population is estimated at nearly 95% white and mestizo (mixed white and Amerindian); 3% Amerindian; and 2% other. Mixtures between the conquering Spaniards, largely Andalusians and Basques, and the Mapuches (Araucanians) produced the principle Chilean racial type (2002 census).

U.S. Black study design and population: A case-control study was initiated by searching clinical databases and bank of biological samples at the University of Pennsylvania and the Perinatology Research Branch (*Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS), at Wayne State University. Study

subjects included U.S. Black women and neonates in the following groups: 1) Cases – women with preeclampsia (*n* = 424) and neonates born to women with preeclampsia (*n* = 375); and 2) Controls – women who delivered at term with a normal pregnancy outcome (*n* = 412) and neonates delivered at term to women with a normal pregnancy outcome (*n* = 462). Race was self-reported and U.S. Blacks were identified by selecting "Black, not Hispanic" on the self-report form. Participants in this study received obstetrical care at the University of Pennsylvania Medical Center, Philadelphia, PA or the Hutzel Women's Hospital, Detroit, MI. The criteria for cases, controls, and exclusion of subjects in the U.S. Black study were the same as described for the Chilean study. Of the maternal and neonatal subjects identified, 78% of samples were identified as maternal-neonatal dyads. To obtain adequate sample sizes for this study, therefore, maternal and neonatal samples were tested independently and un-paired samples were included in each group. The use of clinical data and collection and utilization of maternal blood, cord blood, and neonatal cheek swabs for research purposes was approved by the Institutional Review Boards of the University of Pennsylvania, Wayne State University, the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS, and Virginia Commonwealth University. U.S. Black race was self-reported for all samples.

Clinical definitions: Preeclampsia was defined based on the presence of gestational hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg) and proteinuria (≥300 mg in a 24-hour urine collection, two or more dipstick measurement of 1+, or one or more dipstick measurement ≥2+) according to ACOG (1) and the National High Blood Pressure Education Program (101). Patients were

considered to have a normal pregnancy outcome if they did not have any medical, obstetrical, or surgical complication, and delivered a term neonate (≥37 weeks) of appropriate birth weight for gestational age (102, 134) without complications.

Sample collection: Maternal blood samples were obtained from the mother at the time of enrollment in the protocol. Umbilical cord blood samples or neonate cheek swabs were obtained immediately after delivery. Blood samples were collected with a vacutainer into tubes containing EDTA. The plasma tubes were balanced and centrifuged at 1300*g* for 10 minutes at 4°C to separate cellular components from clear plasma, and the samples were stored at -70°C until assay.

DNA extraction: DNA was extracted from maternal and cord blood with a Qiagen Autopure system using standard procedures (Qiagen). DNA was extracted from neonate check swabs using traditional methods as previously described (135).

Genotyping: Single-nucleotide polymorphism analysis was performed using real-time allelic discrimination TaqMan assays (Applied Biosystems) with modifications. All PCR reactions contained 25-75 ng of DNA, 6.25 ul TaqMan Universal Master Mix (Applied Biosystems) (2x), 0.3 ul TaqMan Genotyping Assay (Applied Biosystems) (20x), and water for a final volume of 12.5 ul. Real-time PCR was performed on an ABI 7500 Fast Real-Time PCR Machine (Applied Biosystems) under the following conditions: 50^{o}C for 2 min, 95^{o}C for 10 min, and 40 cycles of amplification (92^{o}C for 15 sec and 60^{o}C for 1 min). For each cycle, the software determined the fluorescent signal from the VIC- or

FAM- labeled probe (Applied Biosystems). Allelic discrimination for *ERAP2* was performed using TaqMan Genotyping assays C___3282749_20 for SNP rs2549782 and C___25649505_10 for SNP rs17408150 (Applied Biosystems).

Statistical Analysis: Logistic regression in R was used to test for differences in clinical characteristics between disease classes for non-genetic variables. Fisher's exact tests implemented in the PLINK software (103) were used to test individual SNPs for genetic associations with case-control status and to confirm Hardy-Weinberg equilibrium. SNPs with an independent effect were further investigated by multiple logistic regression in R to condition by covariates found to be significantly different between cases and controls in the clinical characteristics analysis. An additive term for the significant SNP(s) was coded as 0, 1, or 2, based on copy number of the minor allele. Allele frequencies from the control groups were used to determine the odds ratios at which our study design had 80% power at an alpha of 0.05. Power calculations were made using the Genetic Power Calculator (122), assuming a 5% disease prevalence.

Results

Clinical Characteristics of the Study Populations: Table 4.1 displays the demographic and clinical characteristics of mothers and neonates from pregnancies with preeclampsia as well as controls. For Chilean subjects, no significant differences were observed in maternal age or fetal sex between groups. Consistent with previous epidemiologic studies, Chilean patients with preeclampsia showed a significantly higher body mass index (BMI) (P <0.001) and fewer previous live births (P = 0.007). In

accordance with preeclampsia resulting in intrauterine growth restriction and indicated preterm birth, offspring born to Chilean women with preeclampsia showed a significantly lower gestational age at delivery and birth weight ($P < 0.001$). Similar results were observed in U.S. Black subjects. Maternal age was not significantly different between cases and controls for either the maternal or the fetal study groups, whereas, gestational age at delivery and birth weight were significantly different between cases and controls for both groups ($P < 0.001$). Additionally, in the fetal group, mothers with preeclampsia showed a significantly higher BMI ($P = 0.049$) and fewer previous live births ($P = 0.040$). Although these measures were not significant in the maternal study group, they were trending in the same direction. In the fetal group, there were significantly more female neonates than male ($P = 0.024$). Significant differences in associations between fetal sex and preeclampsia have been reported in the literature, but results vary with some studies reporting a bias towards male fetuses, some reporting a bias towards female fetuses, and still others reporting no differences in fetal sex in association with preeclampsia (136-144). No significant difference in fetal sex was observed between cases and controls in the maternal study group.

Chilean Population: The minor allele (G) frequencies for rs2549782 in maternal and fetal samples were 0.3386 and 0.3292, respectively. The minor “A” allele frequencies for rs17408150 in maternal and fetal samples were 0.0422 and 0.0395 respectively. The minor allele frequencies are consistent with published data and the Johnson *et al.* study(115, 127). Single SNP analysis revealed no associations between *ERAP2* polymorphisms rs2549782 and rs17408150 and preeclampsia in either maternal or fetal

samples (Table 4.2). All SNPs were found to be in Hardy-Weinberg equilibrium in the maternal and fetal control samples and no substantial linkage disequilibrium was observed (R^2 = 0.087 and 0.072, respectively).

U.S. Black Population: The minor allele (G) frequencies for rs2549782 in maternal and fetal samples were 0.4103 and 0.3990 respectively. The minor allele frequencies are consistent with published data and the Johnson *et al.* study (115, 127). We did not genotype rs17408150 in this population because the minor "A" allele is reported to be < 1.0% in individuals of African descent (115).

To establish the genetic similarity between the University of Pennsylvania Medical Center and Hutzel Women's Hospital U.S. Black samples, and determine if these groups were appropriately combined into a single study population, we compared allele frequencies for three genes: *ERAP2, MTHFR,* and *COMT.* Allele frequencies of both *COMT* and *MTHFR* are not only known to differ among major racial categories, but substantial variation has also been demonstrated in subpopulations of each, including U.S. Black (97, 115-120). Genotypes for *MTHFR* and *COMT* were readily available for our samples and based on their aforementioned racial variation, they represented ideal genes for the genetic comparison of the two U.S. Black sample collection locations. Minor allele frequencies for *ERAP2*, *MTHFR*, and *COMT* were comparable between both U.S. Black study sites (Table 4.3). Additionally, the same *COMT* haplotype structure was identified in each group and the haplotype frequencies were comparable. The genetic similarity of the two groups across six variable SNPs and COMT haplotype

structure and frequency, supported combing the groups into a single U.S. Black study population.

Single SNP analysis yielded a significant association between the fetal rs2549782 and preeclampsia in the U.S. Black population (*P* = 0.009), while no association was observed in the maternal SNP (Table 4.2). Additional multiple logistic regression analysis was performed on the fetal group to adjust for risk factors of preeclampsia (BMI, previous live births, and gravidity) that were found to be significant in the clinical measures analysis (Table 4.4). rs2549782 remained significant (*P* = 0.012) and was associated with an increased risk for preeclampsia (OR = 1.529; CI: 1.099, 2.128). Of the previously identified clinical measures tested, only the number of previous live births remained significant, with a larger number of previous live births decreasing the risk for preeclampsia (OR = 0.845; CI: 0.744, 0.960). All SNPs were found to be in Hardy-Weinberg equilibrium in the maternal and fetal groups. Finally, we used two methods to confirm that the positive association we observed was not attributed to population stratification based on the different U.S. Black sample collection locations. First, multiple logistic regression analysis was performed in R to test whether there was an interaction between the fetal genotype and the sample collection location. No significant association was observed between a location x fetal rs2549782 interaction and the risk for preeclampsia (*P* = 0.098). Second, we performed a cluster analysis in PLINK using a Cochran-Mantel-Haenszel model that tested for overall disease/gene association, while controlling for clusters. After controlling for the sample collection location, the fetal rs2549782 was still significantly associated with an increased risk for preeclampsia (*P* = 0.027; OR = 1.302; CI: 1.029, 1.648). These

results, in addition to the absence of evidence for differences in the rates of preeclampsia between U.S. Black groups in the United States, justifies combining these samples in this study.

Discussion

Preeclampsia is one of the leading causes of maternal and perinatal morbidity and mortality worldwide; yet its etiology is poorly understood (1). It is thought that poor placentation and inadequate maternal blood supply lead to placental hypoxia and the placental release of factors that contribute to intravascular inflammation (30-32), generalized endothelial dysfunction (35, 36, 58-60) and the maternal symptoms. A genetic susceptibility to preeclampsia is well established and genes involved with the immune system, inflammation, hemodynamics, endothelial dysfunction, oxidative stress, and angiogenesis have been associated with preeclampsia (71, 80, 81, 83). The identification of genes involved in a variety of physiologic processes reflects the complex nature of this disorder.

It was recently reported by Johnson *et al*. that the *ERAP2* gene was associated with preeclampsia (127). They found an association with the rs2549782 SNP in an Australian/New Zealand maternal cohort and the rs17408150 SNP in a Norwegian maternal cohort. In the present study, we sought to test whether there were associations between the two previously identified SNPs in *ERAP2* and risk for preeclampsia in two distinct racial sample sets, Chilean and U.S. Black. In contrast to the previous study, we also included fetal samples to determine if the fetal *ERAP2* gene was associated with risk for preeclampsia. We were motivated to use this design by the

fact that placental tissue is of fetal origin and by interest in determining if any genetic association might be attributed to the sharing of alleles between mother and fetus of one-half, in accordance with Mendelian segregation patterns. We found that, in U.S. Blacks, the presence of the minor allele (G) of the rs2549782 SNP in the fetal *ERAP2* gene increased the risk for preeclampsia. We found no associations between the two SNPs in the Chilean population, or the rs2549782 SNP of the maternal *ERAP2* gene in the U.S. Black population.

ERAP2 is a member of the oxytosinase subfamily of M1 aminopeptidases, along with ERAP1 and oxytosinase (49, 50). It catalyzes the cleavage of amino acids, sequentially, from the amino terminus of a variety of protein and peptide substrates (130). ERAP2 is a soluble protein that is expressed ubiquitously throughout the body. It is primarily localized in the endoplasmic reticulum lumen, but under certain conditions, it is also secreted (49, 50). Although aminopeptidases cleave a variety of residues, ERAP2 shows specificity for basic amino acids, and in particular, it preferentially cleaves arginine and lysine (49, 50). ERAP2 has been found to cleave angiotensin III, kallidin, and various N-terminal extended precursors to Major Histocompatibility Complex (MHC) class I- presented antigenic peptides (50). In contrast to other members of this subfamily of enzymes, it does not cleave oxytocin, vasopressin, or angiotensin II (50). As one of the newer aminopeptidases to be described, the full repertoire of substrates for ERAP2 needs to be defined (50).

Preeclampsia is usually diagnosed after 20 weeks of gestation, but it is thought that problems arising early in pregnancy, especially during placentation, are the origin of this disorder. *ERAP2* is expressed in the syncytiotrophoblast and it has been reported

that expression of this gene was down-regulated in first trimester placentas of women who subsequently developed preeclampsia (128, 133). The identification of aberrant gene expression, before maternal symptoms develop, suggests a role for *ERAP2* early in the disease course.

ERAP2 has the potential to contribute to the development of preeclampsia in multiple ways due to its involvement in the regulation of immune responses, pro-inflammatory cytokine production, and blood pressure (49, 50, 130-132). Preeclampsia is associated with a predominant T Helper Cell Type 1 (Th1) immune response, which correlates to poor placentation, inflammation, and endothelial dysfunction (48). One of the primary roles of ERAP2 is Human Leukocyte Antigen (HLA) trimming of class 1-binding peptides. Decreased levels of HLA-G have been reported in the circulation of women with preeclampsia and reduced cell-surface expression has been reported in trophoblasts (49-52). Interferon-gamma (IFN γ) regulates both the *ERAP2* and *ERAP1* genes and they have been implicated in immune activation and inflammation (132). ERAP1, which is closely related to and forms complexes with ERAP2 (51), also cleaves the cell surface receptors for pro-inflammatory cytokines.

Pregnancy is a pro-inflammatory state, and inflammation is a key regulator of placentation (31, 32, 42, 43). Although normal pregnancy is pro-inflammatory, preeclampsia is associated with an exaggerated state of systemic inflammation, and aberrant production of placental cytokines has been widely reported (44). The placental release of pro-inflammatory cytokines, or the pre-existence of increased inflammation in the maternal vasculature, could both contribute to the development of preeclampsia. In addition to being pro-inflammatory, many cytokines also regulate other processes that

are important to the establishment and maintenance of pregnancy. Placentation is tightly regulated by the oxygen balance to ensure adequate remodeling of the maternal spiral arteries and sufficient perfusion of the placenta (34). Hypoxia Inducible Factor 1α (HIF-1α) is a transcription factor that mediates cellular responses to hypoxia and its expression is altered in preeclampsia (33, 62, 145). HIF-1α is regulated through oxygen dependent and independent mechanisms, and several of the cytokines that are modulated by ERAP2 have been shown to participate in the oxygen independent regulation mechanisms (146).

Finally, ERAP2 regulates blood pressure through the renin-angiotensin (RAS) pathway. Specifically, ERAP2 cleaves Angiotensin III and kallidin, both of which are involved in regulating the dilation and constriction of blood vessels (50). Abnormalities in the processing of these vasoactive substances could be a cause of maternal high blood pressure, but they also might participate in placental hypoxia, which is a key feature of preeclampsia. Defects in the RAS system have been demonstrated both in the maternal system and fetal tissue (63, 64), further emphasizing the potential for *ERAP2* to be involved in the pathophysiology of preeclampsia.

Compared to white women (defined as not U.S. Black, Asian, Hispanic, or Native American), Caughey *et al.* found higher rates of preeclampsia among U.S. Black women and lower rates among Hispanic women (66). Additionally, maternal-paternal racial discordance was reported to be associated with an increased incidence (66). This supports the hypothesis that the genetic basis for preeclampsia is heterogeneic. Our results, in conjunction with the findings of Johnson *et al.*, provide a potential explanation for the observed differences between racial groups (127). Four racial populations were

examined between the two studies. Allelic variation between European groups, especially Mediterranean, central Europe, and Scandinavia are well characterized and support that they are distinct populations (97, 115-120). The Chilean population is representative of a Mediterranean racial background, specifically from Spanish descent. *ERAP2* appears to contribute to the risk for preeclampsia in three of the racial groups, with two different allelic variants being associated with risk. Maternal variants increase the risk for preeclampsia in an Australian/ New Zealand cohort and a Norwegian cohort (127). Although preeclampsia is thought to be a placental disorder, the maternal phenotype and, in particular, the susceptibility of the maternal system to disease plays an important role in this disorder (21). Chronic hypertension, obesity, diabetes, and renal disease, all put a woman at increased risk of developing this disorder. A fetal variant increases the risk for preeclampsia in the U.S. Black cohort. Importantly, the placenta is fetal tissue and our results strengthen the argument that primary defects in the placenta play a central role in the development of preeclampsia. Moreover, this finding is consistent with the observation of altered *ERAP2* expression in placentas from women who developed preeclampsia.

One strength of our study is the inclusion of both maternal and fetal genotypes, which gives us the ability to discriminate between maternal and fetal genetic effects. The mother and fetus share fifty percent genetic identity so failure to include both maternal and fetal genes in a study creates the potential for a true association with the unmeasured gene to manifest as an observed association with the measured gene based on the correlation between maternal and fetal genotypes. There is also the potential for both the maternal and fetal *ERAP2* genes to contribute to the risk for

preeclampsia in a single racial population. By measuring only the maternal genes, an additional fetal association could be missed. Thus, the question still remains whether both maternal and fetal *ERAP2* contribute to preeclampsia in different racial populations where only maternal genes were tested.

A second potential source of variation between races is the finding that two different SNPs in the *ERAP2* gene are associated with risk for preeclampsia. Both of these SNPs are missense mutations that are predicted to alter the three-dimensional structure of the protein and damage function. Additionally, rs2549782 resides within the highly conserved zinc-binding domain. While both SNPs are expected reduce enzyme function, they likely alter function to different degrees and are not equivalent mutations. Moreover, the SNPs reside in different domains of the protein and because ERAP2 has multiple functions, the mutations could have significantly different physiologic consequences.

Alternatively, the observed variation could be explained by differences in linkage disequilibrium (LD) structure between populations or failure to account for larger haplotype structure. Although, the SNPs tested in these studies are predicted to alter enzyme function, they might not represent the causal variant in preeclampsia. These populations might share the same causal variant, but that variant could be in LD with different SNPs in each population. Finally, two haplotypes of *ERAP2* have recently been described that lead to changes in mRNA decay and ultimately MHC class I presentation on cell surfaces (147). The haplotypes are composed of numerous SNPs, with rs2549782 representing one of the four coding SNPs that are considered diagnostic (147). The frequency of each haplotype was estimated to be 0.5 across multiple racial

groups and similar patterns of long-range LD were also observed; indicating a single ancestral division of functional significance (147). Neither our study, nor Johnson et al. included the depth of sequencing necessary to characterize the reported haplotypes.

Our findings did not support a genetic association between *ERAP2* and the risk for preeclampsia in either the Chilean population or the maternal U.S. Black population. However, it should be noted that the present study had limited statistical power to detect very small effects. In the Chilean population, our study was adequately powered to detect Odds Ratios of at least 2.3 for rs17408150 and 1.5 -1.7 for rs2549782. In the U.S. Black population, our study was adequately powered to detect Odds Ratios of 1.6 - 1.9 for rs2549782. The effect sizes for a single risk factor in a complex disorder are expected to be relatively modest. Furthermore, we only tested for associations between two SNPs in the *ERAP2* gene so we are unable to rule out the possibility that different variants of this gene are associated with risk for preeclampsia in these populations. Future studies, increasing the number of markers to saturate the maternal and fetal *ERAP2* genes, are needed to characterize the haplotype structures of each group in order to distinguish between maternal and fetal effects of this gene.

Conclusions

Our results show that fetal carriage of the minor allele (G) of rs2549782 in the *ERAP2* gene increases the risk for preeclampsia in U.S. Blacks. We found no associations between the maternal rs2549782 SNP of the *ERAP2* gene and risk for preeclampsia in either the U.S. Black or Chilean populations or the rs17408150 SNP of the *ERAP2* gene and risk for preeclampsia in the Chilean population. The association of

rs2549782 with risk for preeclampsia is consistent with findings of a previous study that found an association of maternal *ERAP2* alleles in an Australian/New Zealand population (127). The results of our study, in combination with those of Johnson *et al*. (127), describe replicated associations between *ERAP2* and preeclampsia in three distinct populations. These observations represent an important step in understanding the pathophysiology of preeclampsia and how genetic variation might play a significant role in racial differences.

Table 4.1. Maternal and fetal characteristics of pregnancies diagnosed with preeclampsia and controls.

Population		Preeclampsia	Controls	*P*-value
Chilean Maternal-Fetal Dyads	Number of dyads	528	575	-
	Maternal Age (*years)*	26.3 (7.5)	26.1 (6.2)	0.692
	BMI (*kg/m^2)*	26.4 (5.4)	24.5 (4.4)	< 0.001
	Previous live births	0.80 (1.19)	0.99 (1.08)	0.007
	Birthweight (*grams)*	2805.7 (815.7)	3423.2 (303.0)	< 0.001
	Gestational age at delivery (*weeks)*	36.8 (3.4)	39.7 (1.1)	< 0.001
	Fetal sex (*% female)*	45.8	53.3	0.492
U.S. Black Maternal	Number of subjects	424	412	-
	Maternal Age (*years)*	26.0 (6.3)	25.3 (5.9)	0.100
	BMI (*kg/m^2)*	30.9 (8.7)	29.7 (7.9)	0.070
	Previous live births	3.2 (2.3)	3.3 (2.0)	0.529
	Birthweight (*grams)*	2431.1 (893.8)	3292.1 (462.4)	< 0.001
	Gestational age at delivery (*weeks)*	36.0 (3.7)	39.5 (1.3)	< 0.001
	Fetal sex (*% female)*	52.4	48.4	0.253
U.S. Black Fetal	Number of subjects	375	462	-
	Maternal Age (*years)*	25.8 (6.5)	25.8 (6.1)	0.947
	BMI (*kg/m^2)*	31.0 (8.5)	29.8 (7.9)	0.049
	Previous live births	3.1 (2.2)	3.4 (2.1)	0.040
	Birthweight (*grams)*	2490.3 (851.8)	3294.7 (469.7)	< 0.001
	Gestational age at delivery (*weeks)*	36.2 (3.4)	39.5 (1.2)	< 0.001
	Fetal sex (*% female)*	54.8	47.0	0.024

Data are presented as means (SD). BMI, body mass index.

Table 4.2. *ERAP2* Allelic analysis for maternal and fetal samples with and without preeclampsia.

Population	*ERAP2* SNP	Genotype (count)	Minor Allele	Frequency Preeclampsia	Frequency Controls	*P*-value	Odds Ratio (95% C.I.)
Chilean	Maternal						
	rs2549782	GG (135) TG (477) TT (491)	G	0.330	0.347	0.393	0.925 (0.775, 1.104)
	rs17408150	AA (2) TA (89) TT (1012)	A	0.044	0.041	0.752	1.069 (0.706, 1.619)
	Fetal						
	rs2549782	GG (124) TG (477) TT (500)	G	0.333	0.326	0.751	1.033 (0.865, 1.234)
	rs17408150	AA (0) TA (87) TT (1014)	A	0.040	0.039	1.000	1.021 (0.665, 1.568)
African American	Maternal						
	rs2549782	GG (147) TG (383) TT (295)	G	0.429	0.391	0.133	1.166 (0.958, 1.420)
	Fetal						
	rs2549782	GG (114) TG (387) TT (268)	G	0.435	0.369	0.009	1.320 (1.075, 1.619)

SNP, single nucleotide polymorphism; C.I., confidence interval. The minor allele (G) of rs2549782 was found significantly more frequently in cases than controls in U.S. Black fetal samples.

Table 4.3. Genotype and Haplotype frequencies for *ERAP2, MTHFR,* and *COMT* for U.S. Black samples.

				Minor Allele Frequency	
Group	Gene	SNP/Haplotype	Minor Allele	Pennsylvania	Michigan
Maternal	*ERAP2*	rs2549782	G	0.397	0.374
	MTHFR	rs1801133	T	0.112	0.102
	COMT	rs6269	G	0.393	0.384
		rs4633	T	0.292	0.316
		rs4818	G	0.200	0.215
		rs4680	A	0.276	0.291
		ATCA		0.249	0.270
		GCCA		0.027	0.021
		GCGG		0.163	0.169
		ACGG		0.037	0.048
		ATCG		0.043	0.045
		GCCG		0.203	0.194
		ACCG		0.278	0.253
Fetal	*ERAP2*	rs2549782	G	0.359	0.424
	MTHFR	rs1801133	T	0.120	0.133
	COMT	rs6269	G	0.408	0.425
		rs4633	T	0.319	0.300
		rs4818	G	0.214	0.167
		rs4680	A	0.292	0.308
		ATCA		0.267	0.257
		GCCA		0.022	0.051
		GCGG		0.175	0.139
		ACGG		0.040	0.028
		ATCG		0.049	0.043
		GCCG		0.207	0.235
		ACCG		0.240	0.247

U.S. Black samples originated from two locations: the University of Pennsylvania Medical Center, PA and Hutzel Women's Hospital, MI. Minor allele frequencies and haplotype frequencies were calculated from control samples only at each location. When comparing locations, no test achieved a significant difference at the 5% level using a Z-test for differences in two independent proportions. SNP, single nucleotide polymorphism. *COMT* single SNP frequencies are listed first, followed by *COMT*

haplotypes formed by those SNPs. *COMT* haplotype SNP order: rs6269, rs4633, rs4818, rs4680.

Table 4.4. Logistic regression model for preeclampsia, including presence of the rs2549782 minor allele in U.S. Black fetuses.

Term	Estimate (S.E.)	*P*-value	Odds Ratio (95% C.I.)
Fetal rs2549782	0.425 (0.169)	0.012	1.529 (1.099, 2.128)
Maternal BMI	0.021 (0.014)	0.140	1.021 (0.993, 1.049)
Previous live births	- 0.168 (0.065)	0.010	0.845 (0.744, 0.960)
Fetal Sex (% female)	0.355 (0.228)	0.120	1.426 (0.912, 2.231)
Intercept	-1.810 (0.492)	< 0.001	-

S.E., standard error; C.I., confidence interval; BMI, body mass index. The minor allele (G) of rs2549782 significantly increases the risk for preeclampsia in U.S. Black fetal samples; after correcting for risk factors identified to modulate risk in this population.

Chapter 5: Separating the genetic and environmental risks for preeclampsia in White and Black Women from the United States.

Introduction

Preeclampsia is considered a complex disorder with multiple genetic, environmental, and social factors contributing to the disease (21, 22). Epidemiologic studies have consistently shown that first degree relatives of women with preeclampsia and daughters from pregnancies complicated by preeclampsia are at increased risk for developing the disorder (71-75). Moreover, sons from preeclamptic pregnancies are more likely to conceive pregnancies with preeclampsia (77). Large twin studies have estimated the heritability for preeclampsia to account for approximately half of the variance attributed to the disorder (0.54; 95% CI, 0-0.71) (76, 78). Consistent with the hypothesis that preeclampsia is a disease of placental origin with maternal contributions to disease, both maternal and fetal genetic components are predicted to contribute to the heritability. Cnattingius estimated the respective maternal and fetal contributions to be 0.35 (95% CI, 0.33-0.36) and 0.20 (95% CI, 0.11-0.24) (78). Although these estimates are widely accepted, they have several limitations. The heritability estimate for preeclampsia was not statistically significant based and had wide confidence interval. Furthermore, the study design used to separate the maternal and fetal genetic components was under-identified and did not include enough unique familial relationships to estimate the four variance components (fetal genetic, maternal genetic,

shared environment, and unique environment). Finally, these studies were based on large Swedish cohorts, and therefore only provide estimates for Scandinavian Whites.

Differences in incidence and severity of preeclampsia exist among different racial populations. Black women from the United States (U.S.) have higher rates of preeclampsia and more severe disease compared to U.S. Whites and U.S. Hispanics (65-68). Furthermore, U.S. Black women and fetuses have higher case-fatality rates compared to Whites and Hispanics (3, 70). These racial differences cannot be explained solely by differences in socioeconomic indicators (67, 68). Despite the racial disparity and resulting significant increases in maternal and fetal morbidity and mortality from preeclampsia for U.S. Blacks, most research has focused on White women, and has not included additional racial populations. Importantly, the heritability of preeclampsia has not been studied in Blacks, so the contributions of genetic and environmental factors to disease have yet to be determined.

Recently, York *et al.,* proposed a new study design that allowed for the separation of fetal genetic, maternal genetic, shared environment, and unique environment variance components for preterm birth (148, 149). By using children of twins (COT), supplemented with full and half-sibling relationships, they were able to include familial relationships that varied in the correlation of fetal genetics, maternal genetics, and shared environments. These relationships were then used to estimate the contributions of fetal genetics, maternal genetics, shared environment, and pregnancy specific environment to preterm birth (149). Moreover, York *et al.* demonstrated that differences in variance components existed between U.S. Whites and U.S. Blacks for

preterm birth (149). This extended twin design, provides a powerful tool for separating the genetic and environmental factors contributing to pregnancy-specific disorders.

In the current study, the COT, supplemented with full and half-siblings, design was used to determine the common fetal, maternal, shared environment, and unique environment variance components underlying preeclampsia for Whites and Blacks. This analysis revealed that racial differences existed in the extent to which the prevalence of preeclampsia declined across subsequent births. Moreover, the analysis showed preeclampsia could not be characterized by a common set of genetic and environmental factors contributing equally across birth order. Differences across birth order necessitate a new modeling approach that allows for additional birth order-specific factors or influences between pregnancies.

Methods

Study Population: The sample population was obtained by combining the birth records from 1989 to 2008 from the Virginia Department of Health (VDH) Office of Vital Records for full and half-sibships with birth records identified through the Mid-Atlantic offspring Twin Registry (MATR), as previously described (149). Informed consent was not required since personally identifiable information was not submitted by either the MATR or VDH. Birth outcome exclusion criteria included multiple births, any congenital anomalies, maternal hemoglobinopathies, and gestational ages > 45 weeks or < 20 weeks. For each birth record, race was classified as Black if the child's race and the race of both parents were listed as non-Hispanic Black and White if the child's race and the race of both parents were listed as non-Hispanic White. After screening, the sample

used in this study consisted of 766,811 births, of which 17.6% were classified as Black (Table 5.1).

Model for Maternal and Fetal Effects: Expectations for genetic and environmental contributions to variances and covariances of relatives were derived from biometrical genetic theory, as previously described (149), and are summarized in Table 5.2. Structural equation modeling was used to derive estimates of genetic and environmental effects using software that implements maximum likelihood approaches (150). These methods yield goodness-of-fit indices quantifying how well the model accounts for the empirical variances and covariances and enabling the testing of hypotheses regarding the causes of variation within groups and their heterogeneity between groups.

Parameter Estimation and Hypothesis-Testing: Expectations for covariance matrices were specified for each sibship and children of twins family type based on the equations in Table 2. A model assuming common genetic and environmental influences (common factor) across pregnancies was used. The model specifications were adapted from York *et al.* for a binary outcome (149). For the binary outcome, a constraint was added, which required the sum of the variances ($f^2 + m^2 + hc^2 + e^2 + (c^2 - hc^2)$) to equal one. Model assumptions included: (1) random mating; (2) genetic effects were additive and constant over pregnancies; (3) the influence of fetal and maternal genetic differences are the same for male and female fetuses (*i.e.,* genetic effects are autosomal and neither X-linked nor sex-limited); (4) genetic and environmental variables do not interact

and; (5) environmental effects were pregnancy-specific apart from the effects of maternal genotype, shared environmental effects, measured covariates, and other aspects of the parental phenotype (*e.g.,* cultural inheritance).

Maximum likelihood estimates of the thresholds and expected covariance matrices were obtained using the structural equation modeling program Mx (150). To ensure that models fully converged and that maximum likelihood estimates were reached, three million iterations were used. Furthermore, the models were re-run two additional times with the same model specifications, but starting the next model from the values determined by the previous analysis. The final estimates were reported from the results of the third model. A test of heterogeneity was performed by equating the thresholds and genetic and environmental parameters across racial groups, assessing the decline in model fit. The optimum number of thresholds needed was determined by observing the decline in fit as thresholds were equated both within and between racial groups. The contribution of individual parameters were examined by dropping each in turn from the model and observing the decline in fit of the submodel by the likelihood ratio chi-square test and change in the Akaike Information Criterion (AIC) in an attempt to arrive at a model yielding the optimal balance of parsimony and goodness-of-fit. Measured covariates were included for a sub-analysis of the European American population to determine if they could clarify the results of the full model. Two covariates were chosen based on prior evidence of association with preeclampsia to account for differences between births: maternal age and birth order. Both extremely young age and advanced maternal age are risk factors for preeclampsia, so the square of the estimate

for maternal age was used as the covariate in the threshold equations. Birth order was coded as a factor with values 1 to 4.

Results

Table 5.3 summarizes model-fitting statistics for multiple models to determine the number of thresholds needed within and between racial groups and whether racial groups could be equated. The full model (model 1 in table 5.3) allowed for separate thresholds for each birth and for the effects of fetal genetic (f^2), maternal genetic (m^2), shared environment (c^2), and unique environment (e^2) to take unique values in each race. The full model also included a term *h,* to allow for differences in the contribution of the shared (familial) environment between full and half-siblings. Compared to model 1, model 2 with *h* removed resulted in a non-significant degradation in model fit and indicated that this parameter could be omitted. All subsequent nested models were compared to model 2. Model 3 indicated that groups could not be equated across race, providing evidence for racial heterogeneity. Models 4 to 6 indicated that thresholds could not be equated across all births for either Whites or Blacks. Models 7 and 8 also show that thresholds could not be equated between races according to birth order, further providing evidence for racial differences in the prevalence of preeclampsia. Model 9 indicated that a separate threshold for each birth was needed for Whites, while model 10 indicated that for Blacks only two thresholds were needed. One threshold was needed for the first birth, but the thresholds for births 2 to 4 could be equated. In addition to 4 thresholds being indicated for Whites, model 11 showed that the thresholds did not have a linear change from 1 to 4.

Model fitting statistics for multiple models for the source and magnitude of factors contributing to variation in preeclampsia and heterogeneity between races are presented in table 5.4. The full model for this analysis was taken from the threshold model analysis (model 10, table 5.3), and included a separate threshold for each White birth and two separate thresholds for Blacks, with one threshold for the first birth and a second threshold for births 2 to 4. In the full threshold model, thresholds, genetic, and environmental parameters were allowed to take on unique values in each race as described for table 5.3. Models 2 to 5 in table 5.4 show that fetal genetic, maternal genetic, shared environment, and unique environment parameters could each be equated across race in models where the other 3 genetic and environmental parameters were allowed to take on unique values. Furthermore, models 6 and 7 indicated that both genetic or both environment parameters could also be equated in a single model where the other two genetic or environment parameters were allowed to take on unique values. However, model 8 demonstrated that all four genetic and environment parameters could not be equated between races in the same model.

The sequential omission of variance components in models 9 and 10 of table 5.4 showed that both fetal (f^2) and maternal (m^2) genetic parameters could be omitted from the European American models. However, model 11 showed that both genetic parameters could not be omitted from the same model, indicating that there is genetic contribution to preeclampsia, but that the models were unable to separate between genetic parameters and/or between genetic and environment parameters. Model 12 indicated that omitting shared environment (c^2) from the White model, resulted in a significant degradation in model fit, thus it could not be omitted. Models 13 to 16

indicated that fetal genetic (f^2), maternal genetic (m^2), and shared environment (c^2) variance components could all be omitted from the Black models. Unlike Whites, both genetic parameters could be omitted from the same model. The ability to omit all parameters from the Black models further suggests that the current models were unable to separate the four genetic and environment variance components, rather than the terms actually being able to be omitted.

Based on the results of table 5.4, a more parsimonious model than the full common factor threshold model, was unable to be reached. Therefore, table 5.5 shows the estimates of thresholds and variance components for the full threshold model. There was a small but statistically significant difference (P = 0.047) in the prevalence of preeclampsia between Whites and Blacks among first births (4.7% and 4.9, respectively). After the first birth, the prevalence of preeclampsia decreased for both races. For Whites the prevalence of preeclampsia decreased successively for each additional birth from 3.0% for the second birth to 2.5% for the fourth birth. However, for Blacks, the prevalence of preeclampsia decreased to 4.1% for the second birth and remained at this level for each additional birth. Thus, the prevalence of preeclampsia for Whites was 39% lower than Blacks by the fourth birth. When all births were combined, the overall prevalence of preeclampsia in Whites was 4.0% and for Blacks was 4.5%. The difference between races in prevalence of preeclampsia for subsequent births indicates that this is the source of the overall higher incidence of preeclampsia reported for Blacks.

The results of table 5.4 also lead us to conclude that reliable estimates for the four variance components could not be determined. Table 5.5 shows the variance

components estimates for the full threshold model as a matter of record. For Whites, fetal genetic factors explain 16.2%, maternal genetic factors explain 11.8%, shared environmental factors explain 25.7%, and unique environmental parameters explain 46.4% of variability of preeclampsia. For Blacks, fetal genetic factors explain 32.4%, maternal genetic factors explain 8.3%, shared environmental factors explain 10.4%, and unique environmental parameters explain 48.9% of variability of preeclampsia. These estimates remained stable across the variety of models tested, which suggests that the models are converging, and that the difficulty with separating factors is not the result of computational limitations or errors.

Because the prevalence of preeclampsia decreased with successive births, risk factors were potentially modified from one birth to the next. Therefore, a sub-analysis of the White population was performed that included maternal age and a term for birth order to determine whether they could help explain the variability between thresholds and clarify common genetic and environmental contributions to preeclampsia. Table 5.6 summarizes the fit statistics for the models that included covariates. Model 1 is the full threshold model with the two covariates. Model 2 indicated that omitting both covariates resulted in a significantly worse fitting model. Models 3 and 4 indicated that birth order could be omitted, but maternal age could not be omitted. Being able to drop birth order was consistent with the observation that a linear term could not be fitted to the thresholds in table 5.3. Models 5 to 7 indicated that with the addition of maternal age, omitting either maternal age or shared environment resulted in a significant degradation in model fit. Although this is an improvement over the results presented in table 5.4, the common factor model is still unable to estimate the fetal genetic contribution to

preeclampsia. This sub-analysis provides further evidence that a common factor model is not an appropriate approach for preeclampsia.

Discussion

Applying the Children of Twins (COT), supplemented with full and half-siblings, structural equation modeling design to preeclampsia yielded new insights into the disorder, but ultimately demonstrated that a common factors model does not accurately model this disease. Preeclampsia research predominantly focuses on the first pregnancy of a woman, but by including up to four births per woman, this study provided an unanticipated source of racial differences. U.S. Black women show higher rates of preeclampsia and this study suggests that the higher rate results from Black women having a smaller decrease in prevalence with subsequent births. The difference in prevalence of preeclampsia in the first births among White women and Black women is small, but statistically significant (4.7% and 4.9%, respectively). Both populations showed the highest rates of preeclampsia in first births, which is consistent with primigravida being at higher risk for preeclampsia (13, 14). However, the prevalence of preeclampsia in White women decreased across subsequent births to a much greater extent than in Black women. The prevalence of preeclampsia in White women decreased with each subsequent birth to 2.5% by the fourth birth. Black women also showed a decrease in prevalence after the first birth, but the prevalence only decreased to 4.1% for the second birth and then stayed constant for births three and four. When all births were considered together the prevalence of preeclampsia was 4.0% in Whites and 4.5% in Blacks, which is consistent with reported values for these populations (67,

68, 70). This suggests that White women are able to decrease their risk for preeclampsia with subsequent pregnancies to a much greater degree than Black women. The reason for this racial difference is unknown, but one possibility is that White women are able to modify their environment or behavior to a greater degree than Black women. Black women may be limited in their ability to change their circumstances and therefore remain at higher risk for subsequent births. For example, Black women may be unable to increase their pre-natal care for subsequent pregnancies, but White women may increase their care with subsequent pregnancies based on their experience during the first pregnancy. Alternatively, the continued high risk in Blacks could be the result of an increase in risk factors over time that offsets an underlying decline. U.S. Blacks have a higher prevalence of chronic hypertension, obesity, and insulin resistance (151-154). These maternal conditions are risk factors for preeclampsia and they increase in prevalence with increasing age. It is possible that the initial risks for preeclampsia decline with subsequent births in a similar pattern between U.S. Whites and Blacks, but that Blacks accumulate more maternal risk factors across subsequent births than Whites.

Although the differences in prevalence across births provided new information on racial differences in preeclampsia, it also presented challenges for the model design that was chosen for this study. York *et al.* demonstrated that a common factor model was able to estimate fetal genetic, maternal genetic, shared environment, and unique environment contributions to preterm birth. Preterm birth has many similarities to preeclampsia including both fetal and maternal genetic contributions (148, 155). As such, it was reasonable to assume that a model similar to the one used for preterm birth

was a good place to begin for preeclampsia. Preterm birth is a continuous variable outcome and the mean could be equated across all four births. Preeclampsia is a binary outcome that requires a threshold model. Unlike preterm birth, preeclampsia required different thresholds for different birth orders. Thresholds are more correlated with the variance components than means are for a continuous variable outcome. This makes the variance components more sensitive to variability in thresholds across births and likely contributed the inability of the common factors model to separate between variance components. More importantly, the necessity of multiple thresholds within each race, established that difference existed between births.

Differences between births led to the common factors model failing to separate variance components within and between races for preeclampsia. Each variance component could be equated across race, yet all could not be equated in a single model. Additionally, within races multiple variance components could be dropped one at a time, but multiple parameters could not be dropped at once. Both of these observations indicated that there were wide confidence intervals around the parameters and that the confidence intervals included zero. The parameters themselves are correlated with each other, as relatives share contributions from up to three components (fetal genetics, maternal genetics, and shared environment). Therefore, when one of these parameters was equated or dropped, the correlated parameters were adjusted within the wide confidence interval to compensate for the model adjustment. This was evidenced by the ability to drop one genetic term at a time from the White models, but not both genetic terms from a single model. The results indicated that the current model was not appropriate for preeclampsia.

Failing to separate parameters and the wide confidence intervals around the estimates mimics the results expected from an underpowered study. This study included large numbers with more than 600,000 birth for Whites and more than 100,000 births for Blacks. The eight familial relationships and the large number of births provided adequate numbers for estimating the four parameters. This further suggests that the common factor model is an inadequate design for preeclampsia.

The final attempt to improve upon the common factor model was including covariates to potentially account for the differences between births that were observed. Both extremely young age and advanced maternal age are risk factors for preeclampsia and age is a factor that changes across births (4, 14). Additionally, including a term for birth order could account for a linear change across birth order. Consistent with the results for the threshold models, the birth order term did not improve the model. This demonstrated that the difference in birth order was not simply a step-wise change from one birth to the next in U.S. Whites. Maternal age did explain a significant amount of the variation and resulted in thresholds that were more similar across births. However, the model with maternal age was still unable to separate between genetic terms. At this point, the evidence overwhelming indicated that a common factor model was not appropriate for preeclampsia, and no further exploration of this model was warranted.

In spite of the limitations of the common factor model, lessons learned in this analysis can be used to guide the development of new models for estimating the variance components that contribute to preeclampsia. The next approach to modeling preeclampsia will need to focus on how factors change and what factors change between births. There are several models that might be able to address these issues.

First, the change between births may not be linear and simply dependent on birth order. Inter-birth interval may play a role in how the risk for preeclampsia changes with subsequent births. Including a measure of the time between births would allow for non-linear changes within one woman's birth history, and would allow for differences between women with respect to equally ordered births. Second, there may be unique variance components for each birth in addition to underlying common factors for all births. A model that would allow for the distinction and evaluation of both common factors across pregnancies and pregnancy-specific factors might be more appropriate for preeclampsia. Finally, the common factor model assumes that births are correlated, but fails to allow for births to exert influences on each other. The first birth could affect the second birth or all subsequent births. Thus, each birth could be influenced by either the immediately preceding birth, or the additive effects of all previous births. There is good evidence for this in normal pregnancies. With each subsequent birth, labor and delivery generally proceeds more rapidly. This suggests that pregnancies are not independent and a woman's body adapts from one pregnancy to the next. These longitudinal effects could result from changes in any one of the genetic or environmental influences on preeclampsia. For example, maternal genetic factors, such as those that influence chronic hypertension, obesity, or insulin resistance, could increase the percent of disease accounted for by maternal genetics and/or pregnancy-specific environment over time. Additionally, major shifts between total genetic and environmental influences could take place over time. Models to account for longitudinal and "carry-over" effects have been developed and could be adapted to preeclampsia (156).

Table 5.1 Sample Frequencies by parental relationship and race.

	White		Black	
Parental Relationship	**N. Families**	**N. Births**	**N. Families**	**N. Births**
Sibship	290,349	602,860	69,112	123,747
Maternal half-sibship	6,735	12,909	2,431	4,653
Paternal half-sibship	5,419	10,507	2,839	5,542
MZ male twin	622	1,209	71	105
MZ female twin	658	1,336	102	153
DZ male twin	421	779	61	88
DZ female twin	397	767	79	132
DZ male-female twin	990	1,794	160	235
Total	305,591	632,156	74,855	134,655

MZ, monozygotic; DZ dizygotic.

Table 5.2 Expected covariance of preeclampsia expressed as variance components between pregnancy outcomes as a function of relationship between offspring. *Courtesy of Dr. York (149).*

Parental relationship	**Familial relationship of offspring**	**Expected covariance**
MZ female twins	Cousin	$\frac{1}{4} f^2 + m^2$
DZ female twins	Cousin	$\frac{1}{8} f^2 + \frac{1}{2} m^2$
MZ male twins	Cousin	$\frac{1}{4} f^2$
DZ male twins	Cousin	$\frac{1}{8} f^2$
DZ male-female twins	Cousin	$\frac{1}{8} f^2$
Full sibship	Sibling	$\frac{1}{2} f^2 + m^2 + c^2$
Maternal half-sibship	Half-sibling	$\frac{1}{4} f^2 + m^2 + hc^2$
Paternal half-sibship	Half-sibling	$\frac{1}{4} f^2 + hc^2$

f^2 = fetal genetic, m^2 = maternal genetic, c^2 = shared familial environment, h = parameter to allow for differences in half-sibling versus full-sibling shared environment.

Table 5.3 Indices of model fit to determine within-group and between-group threshold parameters.

Model	Description	-2LL	*k*	AIC	*P*-value
1	Full model	246160.50	18	-1287439.5	-
2	Half-sibling environmental *h* parameters omitted from each race	246160.44	16	-1287443.6	0.970
3	No racial heterogeneity	246747.08	8	-1286870.9	<0.001
4	Identical thresholds within each race	247746.13	10	-1285869.9	<0.001
5	Identical White thresholds	247687.94	13	-1285922.1	<0.001
6	Identical Black thresholds	246218.39	13	-1287391.6	<0.001
7	1^{st} threshold equated between races	246164.39	15	-1287441.6	0.047
8	4^{th} threshold equated between races	246170.37	15	-1287435.6	0.002
9	White Identical 2^{nd}-4^{th} thresholds	246173.57	14	-1287434.4	0.001
10	Black Identical 2^{nd}-4^{th} thresholds	246161.34	14	-1287446.7	0.638
11	White thresholds with linear change, Black 2^{nd}-4^{th} thresholds equated	246399.26	12	-1287212.7	<0.001

The table presents the following values: -2 times the log likelihood (-2LL), the number of free parameters in the model (*k*), an index of the balance between goodness of model fit and parsimony (Akaike's information criterion, or AIC), and the *P*-value significance result from the likelihood ration test (distributed as a chi-square statistic). The Full model is a common factor model that allows for the following parameters to take on unique values within each race: four thresholds (1 for each birth), four variance components (fetal genetics, maternal genetics, shared environment, and unique environment), and a term (*h*) to allow for differences between shared environmental contributions for full and half-siblings. The fit of model 2 was compared to model 1 and all subsequent models were compared to model 2. Significant *P*-values indicate a significant reduction in model fit. f^2, fetal effect; m^2, maternal effect; c^2, shared environmental effect.

Table 5.4 Indices of model fit to assess within-group genetic and environmental contributions and between group racial heterogeneity.

Model	Description	-2LL	*k*	AIC	*P*-value
1	Full threshold model	246161.34	14	-1287446.7	-
2	f^2 equated across race	246161.49	13	-1287448.5	0.705
3	m^2 equated across race	246161.44	13	-1287448.6	0.762
4	c^2 equated across race	246162.60	13	-1287447.4	0.263
5	e^2 equated across race	246161.42	13	-1287448.6	0.780
6	f^2 and m^2 equated across race	246161.48	12	-1287450.5	0.932
7	c^2 and e^2 equated across race	246165.62	12	-1287446.4	0.118
8	f^2, m^2, c^2 equated across race	246214.94	11	-1287399.1	<0.001
9	White f^2 omitted	246162.21	13	-1287447.8	0.352
10	White m^2 omitted	246164.31	13	-1287445.7	0.085
11	White f^2 and m^2 omitted	246168.48	12	-1287443.5	0.028
12	White c^2 omitted	246175.00	13	-1287435.0	<0.001
13	Black f^2 omitted	246162.35	13	-1287447.6	0.315
14	Black m^2 omitted	246161.92	13	-1287448.1	0.448
15	Black f^2 and m^2 omitted	246166.62	12	-1287445.4	0.071
16	Black c^2 omitted	246162.19	13	-1287447.8	0.358

The table presents the following values: -2 times the log likelihood (-2LL), the number of free parameters in the model (*k*), an index of the balance between goodness of model fit and parsimony (Akaike's information criterion, or AIC), and the *P*-value significance result from the likelihood ration test (distributed as a chi-square statistic). The fit of all models was compared to model 1. Significant *P*-values indicate a significant reduction in model fit. f^2, fetal effect; m^2, maternal effect; c^2, shared environmental effect.

Table 5.5 Estimated thresholds and variance components from full threshold model.

Population	Source	Estimate	Percent
White	1st threshold	1.673	4.7
	2nd threshold	1.880	3.0
	3rd threshold	1.891	2.9
	4th threshold	1.965	2.5
	Fetal genetic	0.162	16.2
	Maternal genetic	0.118	11.8
	Shared environment	0.257	25.7
	Unique environment	0.464	46.4
Black	1st threshold	1.655	4.9
	2nd threshold	1.741	4.1
	3rd threshold	1.741	4.1
	4th threshold	1.741	4.1
	Fetal genetic	0.324	32.4
	Maternal genetic	0.083	8.3
	Shared environment	0.104	10.4
	Unique environment	0.489	48.9

Table 5.6 Indices of model fit to assess maternal age and birth order as covariates for Whites

Model	Description	-2LL	*k*	AIC	*P*-value
1	Full threshold model with covariates	205238.12	10	-1058323.9	-
2	No covariates	205252.79	8	-1058313.2	<0.001
3	Maternal age omitted	205252.77	9	-1058311.2	<0.001
4	Birth order omitted	205238.16	9	-1058325.8	0.834
5	f^2 omitted	205238.17	8	-1058327.8	0.975
6	m^2 omitted	205244.17	8	-1058321.8	0.049
7	c^2 omitted	205256.64	8	-1058309.4	<0.001

The table presents the following values: -2 times the log likelihood (-2LL), the number of free parameters in the model (*k*), an index of the balance between goodness of model fit and parsimony (Akaike's information criterion, or AIC), and the *P*-value significance result from the likelihood ration test (distributed as a chi-square statistic). The fit of models 2 to 4 was compared to model 1 and the fit of models 5 to 7 was compared to model 4. Significant *P*-values indicate a significant reduction in model fit. f^2, fetal effect; m^2, maternal effect; c^2, shared environmental effect.

Chapter 6: Perspectives

Throughout the course of this dissertation, I have addressed racial differences in the fetal and maternal genetic contributions to preeclampsia. In chapter 2, I presented evidence that *Catechol-O-methyltransferase (COMT)* contributes both maternal and fetal genetic effects to preeclampsia, through single gene and epistatic effects with *Methylenetetrahydrofolate reductase (MTHFR)*. Furthermore, I provided evidence that the combination of maternal *COMT* and fetal *COMT* genes in a single pregnancy is important to this disorder. In chapter 3, I extended the study presented in Chapter 2 to include a U.S. Black population. By comparing the findings in Chileans to U.S. Blacks, I demonstrated that there were ancestral differences in allele frequency for both *COMT* and *MTHFR* and that these differences could contribute to racial differences in preeclampsia. In chapter 4, I presented evidence that the fetal *Endoplasmic reticulum aminopeptidase 2 (ERAP2)* is associated with preeclampsia in U.S. Blacks, but not Chileans. In chapter 5, I applied a unique Children of Twins (COT), supplemented with full and half-siblings, to estimate the overall contributions of fetal genetics, maternal genetics, shared environment, and unique environment to preeclampsia in Whites and Blacks. Through this analysis, I uncovered a unique source of racial differences. A discussion of the general conclusions of our research, the implications of our findings, and questions that remain to be answered is presented below.

Maternal and Fetal Genetics

Our candidate gene studies identified both maternal and fetal genetic variants that are associated with the risk for preeclampsia. This represents a significant contribution to our understanding of preeclampsia because these are among the first studies to identify fetal genetic variants that are associated with the risk for this and other pregnancy disorders. We showed that fetal *COMT, MTHFR, and ERAP2* were all three associated with the risk for preeclampsia in certain populations. Identifying three fetal genes that are associated with risk for disease provides strong evidence to support the role of fetal genetics in the development of preeclampsia and emphasizes the need for more studies to include fetal samples.

By studying the three genes (*COMT, MTHFR, and ERAP2*) in maternal-fetal dyads, we also revealed that the same gene could contribute to preeclampsia differently, depending on whether it is the maternal allele or the fetal allele. This is evidenced by the three main findings in chapter 2: (1) *COMT* haplotype alone was associated with the risk for preeclampsia in mothers, but the combination of *COMT* haplotype by *MTHFR* variant was associated with the risk for preeclampsia in fetuses; (2) low COMT activity, predicted based on *COMT* haplotype, reduced the risk for preeclampsia in mothers, but low COMT activity, predicted based on the interaction between *COMT* haplotype and *MTHFR*, increased the risk for preeclampsia in fetuses; and (3) the combination of maternal and fetal alleles in a single pregnancy was related to preeclampsia status. This complexity would not have been appreciated without the inclusion of fetal samples that were paired with maternal samples.

These findings have important implications for our current understanding of how genes contribute to preeclampsia and for developing therapies to treat this disorder. First, previous studies that only included maternal samples provide incomplete results. Maternal and fetal genomes share fifty percent identity. Therefore, by only analyzing maternal genes, fetal effects could manifest through an observed maternal association or fetal effects could be missed altogether. Studies should be extended to include fetal genes to determine if only maternal effect(s), only fetal effect(s), or if both maternal and fetal effects are present. In regards to developing therapies for preeclampsia, focusing on maternal genetic contributions could fail to identify one half of a genes contribution to disease. This could have serious consequences if a gene contributes to disease differently in the mother and the fetus. Developing therapies that address the maternal genetic affect could be unsuccessful if the implications of fetal genes are not understood or considered.

The candidate gene studies of *COMT, MTHFR, and ERAP2* show an association between these genes and risk for preeclampsia, but they do not provide evidence for the mechanism of action. Future studies need to determine how the genes are contributing to preeclampsia. As outlined in chapter 2, placental hypoxia is a key feature of preeclampsia and placental hypoxia-driven imbalances in angiogenic and anti-angiogenic factors are thought to contribute to endothelial dysfunction in this disorder. 2-methoxyestradiol (2-ME) is generated by COMT and 2-ME inhibits Hypoxia Inducible Factor 1α (HIF-1α), a transcription factor mediating hypoxic responses. Cytotrophoblast invasion and placental vascular development have also been reported to be modulated by 2-ME during hypoxic conditions, and this process was associated with a decrease in

the expression of HIF-1α (84). *Comt*$^{-/-}$ mice develop a preeclampsia phenotype that is reversed by exogenous 2-ME (61). Furthermore, circulating concentrations of 2-ME and placental COMT activity have been shown to be significantly reduced in women diagnosed with preeclampsia (61). Taking into consideration our results, we hypothesize that decreased COMT activity in placentas leads to decreased 2-ME, which in turn leads to a failure to inhibit HIF-1α and/or impaired cytotrophoblastic invasion. This would result in the inappropriate up-regulation of hypoxia-induced genes by HIF-1α. In mothers, decreased COMT activity appears to be protective and we hypothesize that decreased COMT activity in the maternal compartment leads to shunting of 2-ME precursors to the fetal compartment. The increase of substrate could help to compensate for the decreased activity of COMT in the placenta. To test these hypotheses, biologic experiments need to be paired with the genotypic information. Specifically, the following experiments are needed: (1) measure COMT protein and activity levels in maternal plasma and placentas to determine if *COMT* haplotype is associated with differences in COMT activity, (2) measure the protein level and activity level of the ATCA *COMT* haplotype with respect to *MTHFR* variant in order to determine how epistasis between these genes affects COMT activity, (3) measure 2-ME and HIF-1α levels in maternal plasma and placentas to determine if *COMT* and *COMT* x *MTHFR* allelic variation are associated with 2-ME and HIF-1α levels in each compartment separately and how the two compartments compare to each other.

ERAP2 has the potential to contribute to preeclampsia through multiple mechanisms including regulation of blood pressure, pro-inflammatory cytokine production, and immune responses (49, 50, 130-132). With multiple mechanisms

possible, a more exploratory approach to investigate the biologic contribution of *ERAP2* is warranted. Designing studies that pair genetic information with a range of biologic mechanisms would be necessary to determine which functions of ERAP2 are important to the development of preeclampsia. In addition to determining the biologic relevance of ERAP2, more work is needed to determine the functional relevance of the identified genetic variants. One allele of rs2549782 is a missense mutation that is predicted to alter the three-dimensional structure of the protein and damage function (127). Additionally, rs2549782 resides within the highly conserved zinc-binding domain. Functional analysis to determine the change(s) in enzyme activity related to rs2549782 genotype need to be performed. There is also good evidence that rs2549782 is important not because of its functional significance, but because it distinguishes between two forms of ERAP2 that are maintained by balancing selection. Two groups of researchers have recently reported an ancestral division in *ERAP2* that resulted in two branches of the gene (147, 157). The branches can be characterized by haplogroups that are characterized by a large number of SNPs. The two haplogroups of *ERAP2* have been maintained through long-standing balancing selection. Although a large number of SNPs fully characterizes the two forms of *ERAP2*, four diagnostic SNPs and rs2549782 in particular can distinguish between them. Importantly, the two forms show large functional differences with one form effectively representing a null mutant. The consequences of the non-functional protein are significantly decreased MHC class 1 presentation (147). Genotyping additional SNPs to fully distinguish the two forms of *ERAP2* in our samples and pairing this information with measures of MHC presentation

in maternal and placental tissue could provide insight into the biologic significance of our observed association.

A common mechanism could also exist for all of the identified associations. All of the genetic variants identified in these studies encoded amino acid changes to the proteins. Differences in amino acid structures of the proteins could, therefore, be appreciated between maternal and fetal proteins. This sets up the potential for genetic conflict between maternal and fetal genes. If fetal COMT, MTHFR, and/or ERAP2 are exposed to the maternal immune system, the mother could mount a response against the fetal proteins because of their amino acid sequence differences. This immune attack could lead to some of the identified abnormalities in preeclampsia including poor placentation, maternal vascular inflammation, placental/fetal vascular and tissue inflammation, placental/fetal cell death, and imbalances in the fetal levels of these proteins.

Genetic Interactions

Preeclampsia is a complex disorder with multiple genes, environmental, and social factors all contributing to disease (21, 22). Complex traits are traditionally thought to result from the additive effects of large number of genes with small effects, however, there is increasing evidence that gene-gene and gene-environment interactions are important contributors to complex traits (158-161). Our findings in chapter 2, that show that epistasis between the fetal *COMT* and *MTHFR* genes is associated with an increased risk for preeclampsia, provides strong evidence for the inclusion of multiple genes and gene networks in preeclampsia research. No association between fetal

COMT or fetal *MTHFR* was observed, but when the combination of *COMT* haplotype and the rs1801133 SNP of *MTHFR* was analyzed, the interaction between the genes was associated with the risk for preeclampsia. If these genes had been studied independently, this relationship would not have been appreciated. Another example of epistasis in preeclampsia was recently reported by Lim *et al.* (162). They found that epistasis between the -34 T/C polymorphism of *Cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1*) and the rs4680 SNP of *COMT* increased the risk for preeclampsia, above and beyond the risk increase due to the rs4680 SNP alone (162). No association was found between the -34 C/T SNP of *CYP17A1* and preeclampsia when this SNP was studied independently (162).

The importance of studying epistasis between genes is further highlighted by contrasting the results of our study presented in Chapter 2, and the study by Roten *et al. (124)*. We found that the maternal low activity haplotype of *COMT* (ACCG) was associated with a decreased risk for preeclampsia in Chilean women, yet Roten *et al.* found that the ACCG haplotype was associated with an increased risk for recurrent preeclampsia in Norwegian women. This could represent genetic heterogeneity in the contribution of *COMT* to preeclampsia, but it also could result from studying *COMT* variation in isolation, without accounting for the additional genetic, environmental, and social factors that it interacts with to cause preeclampsia.

The concept of opposite associations not being contradictory, but rather indicating the complexity of a disorder, was first proposed by Lin *et al.* and is called the “flip-flop” phenomenon (125). They showed through a series of simulation studies, that associations in opposite directions could be fully explained by failing to correct for gene-

gene interactions and differences in linkage between genomic regions, which is a common difference between different racial populations. Our study, the Lim *et al.* study, and the implications of the Lin *et al*. study highlight the importance of considering the complexity of preeclampsia in future research (123, 125, 162). Research on preeclampsia needs to move beyond single gene studies and start to incorporate gene-gene combinations, gene-networks, and factors that contribute to functional pathways into association tests.

A significant limitation to pregnancy research is the separation between maternal and fetal studies. Pregnancy involves two individuals, mother and fetus, in a single biological system. As a result, pregnancy is a unique condition in which there are two genomes and these genomes have the potential to interact. This adds a unique layer to pregnancy disorders by allowing for genetic interactions between mother and fetus in addition to genetic interactions within each individual. There are seven pairwise genetic interactions that should be considered when studying preeclampsia: maternal gene x maternal gene, maternal gene x maternal environment, fetal gene x fetal gene, fetal gene x fetal environment, maternal gene x fetal gene, maternal gene x fetal environment, and fetal gene x maternal environment. Beyond pairwise interactions, interactions between more than two components could also exist and should ultimately be considered when investigating pregnancy disorders. The genetic interactions could be additive (*i.e.*, the effect of 4 alleles rather than the normal 2 for one individual), synergistic, or conflicting (i.e., the difference between maternal and fetal genes). Genetic conflict is a popular concept that has been the focus of much research on preeclampsia. The classic model for genetic conflict in pregnancy is Rhesus (Rh)

incompatibility, which results in hemolytic anemia of the newborn due to a maternal immune response. Currently, statistical methods to investigate maternal-fetal genetic interactions broadly assume the “Rh model”, which omits main effects of maternal or fetal genes because the only mechanism is an incompatibility that results in immune attack (83). To accurately determine how the maternal and fetal genomes might interact to contribute to preeclampsia, genetic models that make no assumptions about the type of interactions should first be considered. The results of such “full” models should then be used to guide the research towards the type(s) of interactions that contribute to the risk for preeclampsia.

Our studies included both maternal and fetal samples and importantly maternal-fetal dyads. This allowed us to look at both maternal and fetal genetic effects in a single pregnancy and to build a more complete view of genetic contributions to preeclampsia. This was an important advancement in preeclampsia research because it captured the pregnancy unit and it is an important study design to consider in the future. Studying maternal-fetal dyads would allow for the analysis of interactions between the two genomes. The challenge at this point is to develop statistical methods that could separate between main effects of genes, genetic interactions within maternal genes, genetic interactions within fetal genes, and interactions between maternal and fetal genes.

Racial Differences

Racial disparities in preeclampsia place U.S. Black women and fetuses at higher risk of developing this disorder, and at higher risk of complications and/or death from

the disorder, compared to U.S. Whites and Hispanics (3, 65-68, 70). Our studies demonstrated racial differences in associations between specific genes and preeclampsia and in the decrease in prevalence of preeclampsia across subsequent births. These findings represent and important advancement in our understanding of the factors that contribute to racial differences in preeclampsia.

There is a genetic contribution to preeclampsia and based on ancestral differences in racial genetic backgrounds, this raises the possibility that genetic differences between populations contributes to differences in preeclampsia. We showed that SNP minor allele and haplotype frequencies of *COMT* and *MTHFR* differed among racial groups. U.S. Blacks showed a looser *COMT* haplotype structure with seven observed haplotypes, compared to three in U.S. Whites and Chileans. Furthermore, the minor allele frequency for the rs1801133 SNP of *MTFR* was only ~0.11 for U.S. Blacks, compared to ~0.45 for Chileans. In our Chilean study we found the maternal *COMT* and an interaction between the fetal *COMT* and *MTHFR* to be associated with the risk for preeclampsia. In our U.S. Black study we found no associations between *COMT and MTHFR* and the risk for preeclampsia in either maternal or fetal samples. The differences in *COMT* haplotype structure/frequency and in minor allele frequency of the rs1801133 SNP of *MTHFR* resulted in a lack of power in the study of the U.S. Black population. We had inadequate (≤ 20%) power to detect effects with the same odds ratios estimated in the study of the Chilean population. As a result, we were unable to determine whether these genes were associated with the risk for preeclampsia in U.S. Blacks. However, the low minor allele frequency of the rs1801133 SNP of *MTHFR* suggests that this variant is of less importance to disease risk in the U.S. Black

population. Future studies need to increase the sample size of the U.S. Black population and increase the number of markers in these genes to determine whether additional variants are more functionally relevant in this population.

We found that the fetal rs2549782 SNP of *ERAP2* was associated with the risk for preeclampsia in U.S. Blacks, but not Chileans. We also found that the maternal rs2549782 SNP of *ERAP2* was not associated with preeclampsia in either population and the rs17408150 SNP of fetal and maternal *ERAP2* was not associated with the risk for preeclampsia in Chileans. An association between the maternal rs2549782 SNP of *ERAP2* and preeclampsia has been previously reported in an Australian/New Zealand population (127). Additionally an association between the maternal rs17408150 SNP in *ERAP2* and preeclampsia in a Norwegian population was reported by the same group (127). rs17408150 has a minor allele frequency of <1% in U.S. Blacks and was, therefore, not included in our analysis of this population. Collectively, these reports and our results demonstrate differences in associations between *ERAP2* and risk for preeclampsia among four different distinct racial populations. The difference in the allele frequencies of SNPs in *ERAP2* documents ancestral differences in variants of this gene between populations. As a result, the differences in association between racial populations could indicate that this gene only contributes to preeclampsia in certain populations, that different polymorphisms in *ERAP2* are associated with disease in different populations, or that linkage between the causative genetic region and the analyzed SNPs differs among the populations. Future studies are needed to increase the number of markers genotyped in maternal and fetal *ERAP2* to be able to determine the source of the differences in association between populations.

We demonstrated racial differences in associations between specific genes and preeclampsia. Another way in which genetic contributions to preeclampsia could differ among racial populations is through differences in percentage of disease explained by genetics within each group. This could be the result of differences in the number of maternal genes, fetal genes, or both maternal and fetal genes that contribute to disease. We hypothesized that variance components modeling would allow us to compare the maternal and fetal genetic contributions to preeclampsia between U.S. Whites and U.S. Blacks. Ultimately, the common factors model we chose proved to be inadequate to model preeclampsia, but it did provide unique insight into racial differences.

An unanticipated discovery in our research was that the difference in prevalence of preeclampsia between U.S. Whites and U.S. Blacks in our study was primarily the result of a greater decrease in prevalence across births for Whites. This provides a unique source of racial differences that had previously not been appreciated. Preeclampsia studies predominantly focus on a woman's first birth, but by including up to four births per woman in our study design, we were able to compare the prevalence of preeclampsia across births. Our study showed only a modest difference in prevalence of preeclampsia between Whites and Blacks for a woman's first pregnancy, but the gap in prevalence widened with each subsequent pregnancy. By the fourth pregnancy, the prevalence of preeclampsia in Whites was 39% lower than in Blacks. When all births were combined, the prevalence of preeclampsia in Whites was 4.0% compared to 4.5% in Blacks. This suggests that the observed increase in incidence of preeclampsia in the U.S. Black population is the result of an increased incidence after

the first birth. Furthermore, the nearly similar rate of preeclampsia seen in the first birth of U.S. Whites and Blacks, also suggests that nulliparous U.S. White and Black women share more risk factors for preeclampsia, than multiparous women. In later births, factors that contribute to risk for preeclampsia in the two populations appear to diverge. Variance components models that can model the change in parameters that contribute to the development of preeclampsia across births are needed to determine the source of the racial difference in prevalence observed in our study.

Variance Components Modeling

A strong genetic contribution to preeclampsia is well accepted, with both maternal and fetal genes contributing to disease. The heritability of preeclampsia has been estimated to be 0.54, with 0.35 being attributed to maternal genes and 0.20 being attributed to fetal genes (76, 78). Despite the wide acceptance of these estimates, the methods used to determine them had limitations that resulted in wide confidence intervals and a failure to fully separate the maternal and fetal genetic parameters. Furthermore, estimates have only been reported for women of Northern European decent. Thus, better methods are needed to more precisely estimate the maternal and fetal genetic contributions to preeclampsia and different racial groups need to be studied to determine if these parameters change between groups.

In chapter 5 we used a novel Children of Twins (COT), supplemented with full and half-siblings, study design to determine the fetal genetic, maternal genetic, shared environmental, and unique environmental contributions to preeclampsia in U.S. Whites and Blacks. By using relationships between the children of twins, this design utilized

eight unique familial relationships and had increased power to separate between fetal and maternal genetics. York *et al.* first used this design to determine the variance components of preterm birth and reported differences between fetal genetic, maternal genetic, and unique environmental contributions to this disorder between U.S. Whites and Blacks (149). We, therefore, hypothesized that applying a similar model to preeclampsia would adequately model this disorder. However, we found that a common factor model was inadequate for preeclampsia and that differences existed between births within each population. Based on these findings, a new modeling approach utilizing the COT data should be considered for preeclampsia.

The common factor model used in our study assumes an underlying genetic biometrical model. It was reasonable to initially assume a genetic model for preeclampsia based on the strong evidence for a genetic contribution to disease and the results of the preterm birth study by York *et. al. (149)*. One reason that the common factor model could have been inadequate for preeclampsia is that a genetic biometrical model was inappropriate. Modeling preeclampsia should start at the beginning with an unstructured model that allows all variances and covariances allowed to take on unique values in twin and sibling type (i.e., a fully saturated model). Nested models of the fully saturated model can then be compared to determine the most parsimonious model that fits the data. This will remove constraints being placed on the model by preconceived assumptions, and will allow for the development of a model that will best fit the data. A genetic biometrical model is a nested model of the fully saturated model and can be compared to determine if it is a good fit for the data.

The second consideration in developing a new approach to modeling preeclampsia, is the observation that more than one threshold is needed within each race. This implies differences in preeclampsia across birth order and future models will need to determine the implications of these differences. There are multiple ways that ways that changes could occur between births. For example, different factors could be important to preeclampsia for each birth, or each birth could be influenced by either the immediately preceding birth, or the additive effects of all previous births. Gillespie *et al.* has developed a longitudinal model of genetic and environmental influences that could be applied to preeclampsia (156). This model does not have to assume a genetic structure and can be used to inform us on how preeclampsia is changing across time (i.e. across births). This will allow us to distinguish between the effects of one pregnancy on the next, the cumulative effects of all previous pregnancies across time, and the effects of unique factors being introduced with each birth. The longitudinal model, that incorporates changes across time, will also be a nested model of the fully saturated model. By starting from the fully saturated model, a complete analysis of the parameters that influence preeclampsia will be possible and will be fully driven by the data.

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Vita

Lori Diane Hill was born on December 28, 1979 in Lynchburg, VA. She graduated from Heritage High School and the Central Virginia Governor's School in 1998 and attended Virginia Polytechnic Institute and State University for her undergraduate education. In 2002, she earned her Bachelor of Science in Biology with Honors after completing her thesis work entitled: "The expression pattern of Flavonoid 3'Hydroxylase (F3'H) in the flavonoid pathway of *Arabidopsis thaliana*". In that same year, she also earned her Bachelor of Arts in Spanish with Honors. Following her graduation, she did research on the evolution and ecology of pelagic sea birds in the Galápagos Islands. In 2003, she returned to the lab of Dr. Brenda Winkel at Virginia Tech and continued research on the flavonoid pathway. Subsequently, she began working as a research assistant in research and development and eventually rose to Laboratory Operations Supervisor for GeneDx in Gaithersburg, Maryland. Under the guidance of Dr. Sherri Bale and Dr. John Compton at GeneDx, she became well versed in genetic diagnostics and gained invaluable knowledge on how to translate research finding into improved patient care. After her acceptance into the physician-scientist program at Virginia Commonwealth University she moved to Richmond, VA where she has spent the past five years. For the first two years she was enrolled in the medical program, and for the past three years she has been a graduate student of the Molecular Biology and Genetics program under the Human and Molecular Genetics Department. Her dissertation has been under the tutelage of Jerome F. Strauss III, M.D.,PhD. She was inducted into Phi Kappa Beta in 2009 and she has presented posters of her work at the Society for Gynecologic Investigation in 2008 and 2010, the American College of Medical Genetics in 2010, and the National M.D./Ph.D. Student Conference in 2010. In the fall of 2011, she will return to the medical program for her clinical rotations and plans to finish her medical degree in May 2013.

List of Publications

Hill LD, York TP, Kusanovic JP, Gomez R, Eaves LJ, Romero R, Strauss JF 3rd. 2011 Epistasis between COMT and MTHFR in maternal-fetal dyads increases risk for preeclampsia. *PLoS One* 6(1):e16681

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