INVESTIGATIONS INTO THE GENETIC SUSCEPTIBILITY TO PRETERM BIRTH

By

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To my parents and sisters for always believing in me
Karl, Digna, Ivette, and Wendy

To my best friend Todd L. Edwards for his love and support

And to my best canine friend and enthusiastic supporter
Bella

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AA	African American
ACE	angiotensin I converting enzyme isoform 1
ADD1	adducin 1 (alpha) isoform a
ADRB2	adrenergic beta-2 receptor
AF	amniotic fluid
ANOVA	analysis of variance
BMI	body mass index
bp	base pairs
C	Caucasian
CARD15	NOD2 protein
CBS	cystathionine-beta-synthase
CCL2	small inducible cytokine A2
CCL8	small inducible cytokine A8
CD14	CD14 antigen precursor
CEPH	Centre d' Etude de Polymorphism Humain
COL1A1	alpha 1 type I collagen preproprotein
COL1A2	alpha 2 type I collagen
COL3A1	procollagen type III alpha 1
COL5A1	alpha 1 type V collagen preproprotein
COL5A2	alpha 2 type V collagen preproprotein
CRH	corticotropin releasing hormone

CRHBP	corticotropin releasing hormone binding protein
CRHR2	corticotropin releasing hormone receptor 2
CRP	C reactive protein
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CV	cross validation
CVC	cross validation consistency
CYP19A1	cytochrome P450, family 19
CYP2D6	cytochrome P450, family 2, subfamily D,
DHEAS	dehydroepiandrosterone sulfate
DHFR	dihydrofolate reductase
DNA	Deoxyribonucleic Acid
ECM	extracellular matrix
EDN2	endothelin 2
EM	expectation maximization
EPHX1	epoxide hydrolase 1 microsomal (xenobiotic)
EPHX2	epoxide hydrolase 2 cytoplasmic
F	inbreeding coefficient
FAS	tumor necrosis factor receptor superfamily member 6
FDR	false discovery rate
FII	coagulation factor II
FV	coagulation factor V
FVII	coagulation factor VII
g	grams

GSTP1	glutathione transferase
HSD11B1	11-beta-hydroxysteroid dehydrogenase 1
HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7
HSPA14	heat shock 70kDa protein 14 isoform 2
HSPA1B	heat shock 70kDa protein 1B
HSPA1L	heat shock 70kDa protein 1-like
HSPA4	heat shock 70kDa protein 4 isoform a
HWE	Hardy-Weinberg equilibrium
IFNG	interferon gamma
IGF1	insulin-like growth factor 1 (somatomedin C)
IL-10	interleukin 10
IL-10RA	interleukin 10 receptor alpha
IL-10RB	interleukin 10 receptor alpha
IL-15	interleukin 15
IL-18	interleukin 18
IL-1A	interleukin 1 alpha
IL-1B	interleukin 1 beta
IL-1R1	interleukin 1 receptor 1
IL-1R2	interleukin 1 receptor 2
IL-1RAP	interleukin 1 receptor accessory protein isoform
IL-1RN	interleukin 1 receptor antagonist isoform 3
IL-1β	interleukin 1 beta
IL-2	interleukin 2

IL-2RA	interleukin 2 receptor alpha
IL-2RB	interleukin 2 receptor beta
IL-4	interleukin 4
IL-4R	interleukin 4 receptor alpha isoform b
IL-5	interleukin 5
IL-6	interleukin 6
IL-6R	interleukin 6 receptor
IL-8	interleukin 8
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	kilograms
KL	klotho isoform a
LD	linkage disequilibrium
LR	likelihood ratio
MBL2	soluble mannose-binding lectin
MDR	Multifactor Dimensionality Reduction
MIAC	microbial invasion of the amniotic cavity
MMP1	matrix metalloproteinase 1
MMP2	matrix metalloproteinase 2
MMP3	matrix metalloproteinase 3
MMP8	matrix metalloproteinase 8
MMP9	matrix metalloproteinase 9
mRNA	Messenger Ribonucleic Acid
mtDNA	Mitochondrial Deoxyribonucleic Acid

MTHFR	5,10-methylenetetrahydrofolate reductase
MTRR	methionine synthase reductase isoform 2
NCBI	National Center for Biotechnology Information
NFKB1	nuclear factor kappa-B subunit 1
NFKB1	nuclear factor of kappa light polypeptide gene
NFKBIB	nuclear factor of kappa light chain gene enhancer in B cells
NFKBIE	nuclear factor of kappa light polypeptide gene
NIH	National Institute of Health
NOS2A	nitric oxide synthase 2A isoform 1
NOS3	nitric oxide synthase 3 (endothelial cell)
OR	Odds Ratio
PAI1	plasminogen activator inhibitor 1
PCR	Polymerase Chain Reaction
pg/ml	picograms per mililiter
PGEA1	PKD2 interactor, golgi and endoplasmic reticulum
PGR	progesterone receptor
PGRMC1	progesterone receptor membrane component 1
PGRMC2	progesterone membrane binding protein
PLA2G4A	phospholipase A2, group IVA
POMC	proopiomelanocortin preproprotein
PON1	paraoxonase 1
PON2	paraoxonase 2 isoform 1
PPARG	peroxisome proliferative activated receptor

pPROM	Preterm Premature Rupture of the Membrane
PROM	Premature Rupture of the Membrane
PTB	Spontaneous Preterm Birth
PTGER3	prostaglandin E receptor 3 subtype EP3 isoform
PTGFR	prostaglandin F receptor isoform a precursor
PTGS2	prostaglandin-endoperoxide synthase 2 precursor
SCNN1A	sodium channel nonvoltage-gated 1 alpha
SERPINB2	serine (or cysteine) proteinase inhibitor clade
SERPINE1	plasminogen activator inhibitor-1
SERPINH1	serine (or cysteine) proteinase inhibitor clade
SHMT1	serine hydroxymethyltransferase 1 (soluble)
SLC23A1	solute carrier family 23 alpha 1
SLC23A2	solute carrier family 23 alpha 2
SLC6A4	solute carrier family 6 member 4
SNP	Single Nucleotide Polymorphism
sTNF-R1	soluble tumor necrosis factor receptor 1
sTNF-R2	soluble tumor necrosis factor receptor 2
TFPGA	Tools for Population Genetic Analysis
THBD	thrombomodulin
TIMP3	tissue inhibitor of metalloproteinase 3
TLR2	toll-like receptor 2
TLR4	toll-like receptor 4
TLR7	toll-like receptor 7

TLR8	toll-like receptor 8
TNF-α	tumor necrosis factor alpha
tPA	tissue plasminogen activator
TREM1	triggering receptor expressed on myeloid cells
TSHR	thyroid stimulating hormone receptor isoform 2
UGT1A1	UDP glycosyltransferase 1 family, polypeptide A1
VEGF	vascular endothelial growth factor isoform a

CHAPTER I

OVERVIEW

Spontaneous preterm labor leading to delivery (PTB), gestational age less than 37 weeks, is one of the leading risk factors of neonatal morbidity and mortality. Multiple putative environmental risk factors have been identified, including: infection, stress, socioeconomic status, smoking, and nutrition. The exact causes of PTB, however, are not well understood and most cases of PTB cannot be fully explained by any of these suspected risk factors.

Recent studies have found promising associations with candidate genes and PTB and/or related phenotypes (preterm premature rupture of the membrane (pPROM) and histological chorioamnionitis). These studies have focused on either maternal or fetal genetic information and on the role of single nucleotide polymorphisms (SNPs) singly and/or a single gene and PTB. The goal of the studies presented in the following chapters is to both gain greater understanding of the genetic contributions to PTB and identify a subset of genes that best predict pregnancies at risk of PTB. These studies examine multiple candidates in both mother and fetus and address a significant deficiency in the present understanding of the genetic susceptibility to PTB. A retrospective case-control dataset including African Americans and Caucasians was used.

The introduction and background are presented in part A of Chapter II, including both the clinical aspects and epidemiology of PTB. Previous work in the field is

discussed, as well as the approaches used to analyze this complex phenotype. Finally, the hypothesis and specific aims are presented in part B.

To begin assessing the genetic contribution to the disparity in prevalence rates between African Americans and Caucasians, patterns of genetic variation in two established candidate genes, interleukin 6 (IL-6) and its receptor (IL-6R), were analyzed in mothers and fetuses. IL-6 levels in amniotic fluid (AF) samples were determined in a subset of these pregnancies. Chapter III, part A, discusses a small-scale study examining the racial disparity between African Americans and Caucasians in both IL-6 gene profile and IL-6 protein expression. Part B follows up on an association found between a marker in IL-6 and IL-6 AF levels in part A, expanding on both sample size and the number of markers examined. IL-6 and IL-6R were analyzed for single locus allelic and genotypic tests of association, linkage disequilibrium (LD) structure, and haplotype associations. Haplotype tests of association were performed with outcome considered as dichotomous (PTB/term) and continuous (IL-6 cytokine concentration (pg/ml)). African American, Caucasian, maternal and fetal samples were analyzed separately. Significant associations from both dichotomous and continuous haplotype analyses and across geographic populations converged on three regions: in IL-6 markers (rs1800797, rs1800796, and rs1800795); in IL-6R markers (rs4601580, rs4645618, rs6687726, and rs7549338) and IL-6R markers (rs4537545, rs4845625, and rs4845374).

Chapter IV presents the results of a large scale high-throughput candidate gene screen on PTB. Part A discusses the results from the candidate gene screen in Caucasians and part B discusses the results from the candidate gene screen in African Americans. Chapter V discusses the results from the multi-locus analyses of the high-throughput data

presented in Chapter IV. No strong multi-locus associations were observed. However, a promising result was observed in Caucasian maternal samples in genes from the complement and coagulation pathway. This result suggests that an interaction between coagulation factor V (FV) and coagulation factor II (FII) may be functioning in conjunction with an independent main effect by tissue plasminogen activator (tPA).

Previous genetic studies have overlooked the mitochondrial genome/gene(s) as a plausible PTB candidate. Chapter VI discusses a study where we sought to document association between nonsynonymous mitochondrial DNA (mtDNA) variants A4917G, G10398A, and T4216C and PTB. We performed a case-control analysis of these three mtDNA markers and examined their potential interaction with smoking in PTB. Caucasian pregnancies were examined; African Americans were excluded due to low minor allele frequencies in the African American population. Marginally significant single locus effects were observed for marker A4917G and T4216C after adjusting for an interaction with smoking.

PTB is hypothesized to be a disease mediated by cytokines; however, it is likely that no single factor alone adequately explains PTB risk. Rather, it is more probable that coordinated networks of cytokines affect risk. Chapter VII presents a study where we examined the relationships between cytokines/chemokines and related biomarkers associated with PTB in African Americans and Caucasians, separately. The Th1 and Th2 cytokines/chemokines and related biomarkers included in the study were: Th1 cytokines interleukin (IL)-1, IL-8, tumor necrosis factor- (TNF)-α, and TNF soluble receptors 1 and 2 (sTNF-R1 and sTNF-R2); and Th2 cytokines IL-6 and IL-10. Correlations between

biomarkers were calculated; differences between correlations of African Americans and Caucasians, and differences between cases and controls were measured.

Chapter VIII describes the conclusions and future directions.

CHAPTER II

INTRODUCTION, BACKGROUND, AND SPECIFIC AIMS

A. Introduction and Background

Clinical Aspects of Preterm Birth

Significance and public health impact of preterm birth

The prevalence of spontaneous preterm labor leading to delivery (PTB), defined as gestational age less than 37 weeks, was ~12.3% in the United States (U.S.) in 2005 (Martin et al., 2005). 500,000 deliveries in the U.S. annually are preterm, resulting in a substantial economic and public health burden (Martin et al., 2005). The cost of care for preterm infants in the U.S is ~$26 billion per year for both medical and social expenses (Behrman and Butler, 2006). In addition to the economic costs, 68% of deaths among infants without congenital anomalies is related to being born preterm (Goldenberg, 2002). Despite these statistics methods for treatment, prediction, and prevention have not been clearly established or defined (Martin et al., 2003).

PTB infants face higher risks of both death and lifelong disability relative to infants born at term. For example, PTB infants are at increased risk of both congenital and behavioral anomalies, neuropsychological impairment, chronic lung disease, and visual abnormalities (Hack and Fanaroff, 1993;Jacobson and Dutton, 2000). Preterm related diagnoses are the number one cause of neonatal mortality and the number two cause of infant mortality in the U.S. (Demissie et al., 2001;Gonik and Creasy,

1986;Mathews and MacDorman, 2006). The effects in African Americans are substantially more severe with preterm related disorders being the leading cause of infant mortality (Demissie et al., 2001;Gonik and Creasy, 1986;Mathews and MacDorman, 2006).

In the U.S. the PTB rate has been slowly increasing over the last decade from 9.7% in 1990 to 12.7% in 2005 (Hamilton et al., 2007;Martin et al., 2002). The increasing trends in PTB rates are not limited to the U.S. Similar increasing trends have been seen in Europe and across the world including Denmark where a significant increase in PTB has been observed over the last decade (Langhoff-Roos et al., 2006). It is apparent from these statistics that PTB has the potential to become a major global public health problem if women at risk are not identified and interventions are not developed.

Preterm birth definitions

A preterm birth is defined as a delivery completed at a gestational age less than 37 weeks from last menstrual period. Preterm birth differs from a "spontaneous abortion" by the gestational age cutoff used; for example, a delivery less than 20 weeks gestation is termed a "spontaneous abortion" and a delivery greater than 20 but less than 37 weeks gestation is termed a preterm birth (Creasy et al., 2004). Previously newborns born at a weight less than or equal to 2500 grams were also termed "premature", however, this definition was not appropriate. Approximately one-third of neonates less than or equal to 2500 grams are to due restricted growth and are not due to being born preterm. Today neonates less than or equal to 2500 grams are termed low birth weight infants regardless of gestational age. Preterm birth, in the present context, makes no distinction based on gestational weight.

Preterm birth features substantial etiological heterogeneity and as a result subtypes have been defined in medical literature based on clinical presentation. Two clinical subtypes of preterm birth are: spontaneous preterm birth (PTB) (spontaneous onset of labor leading to delivery or labor following preterm premature rupture of the membranes (pPROM)), and medically indicated preterm birth (when maternal and/or fetal indications interrupt a pregnancy at preterm gestations) (Ananth and Vintzileos, 2006). Medically indicated preterm birth follows a medical or obstetric disorder that puts the mother or fetus at risk (i.e. diabetes, maternal hypertension, and placenta previa or abruption) (Creasy et al., 2004). Spontaneous preterm birth, in contrast to medically indicated preterm birth, arises in the absence of maternal or fetal illness. The studies described in the following chapters describe experiments on the spontaneous clinical subtype of preterm birth following spontaneous onset of labor.

The risk of subsequent preterm births following the occurrence of a preterm birth varies by clinical subtype, but a predisposition to deliver preterm has been seen in both medically indicated and spontaneous preterm birth (Ananth et al., 2006). Among the known risk factors for medically indicated preterm birth (Table 2-1a) proteinuria ranks as the most significant with an Odds Ratio (OR) of 5; however, the strongest risk factor for spontaneous preterm birth is multiple gestations (OR = 6) (Table 2-1b). On average, however, the risk factors for medically indicated preterm birth incur stronger risk (Goldenberg and Rouse, 1998;Meis et al., 1995;Meis et al., 1998). The etiology underlying clinical subtypes varies considerably across both racial and geographic populations, and a higher rate of both subtypes has been observed in African Americans relative to other racial groups (Ananth and Vintzileos, 2006).

Table 2-1. Risk factors for spontaneous and medically indicated preterm birth

a.

Risk Factors Spontaneous Preterm Birth	Odds Ratio
Multiple gestation	6
History of PTB	4
2nd trimester bleeding	2 or >
Genitourinary tract infection	2
African American	2
Age < 18	2
Low BMI	2
Cigarette smoking	1.5
Frequent contractions	1.5

b.

Risk Factors Medically Indicated	Odds Ratio
Proteinuria	5
High blood pressure	4
History of stillbirth	3.5
Lung disease	2.5
Age > 30	2.4
Bacteriuria	2

*Data from (Goldenberg and Rouse, 1998;Meis et al., 1995;Meis et al., 1998)

Diagnosis and treatment

Diagnosis. Upon a pregnant woman's admission into a hospital, labor is detected by digital examination and diagnosed by the presence of persistent uterine contractions with dilation and effacement of the cervix (Gonik and Creasy, 1986). Ultrasound and last menstrual period dating are commonly used to determine if a delivery occurs preterm. However, recently in efforts to reduce false positive preterm labor diagnoses, fetal fibronectin tests from cervicovaginal fluid have also been used to diagnose the onset of labor. Fibronectin is a protein from the extracelullar matrix (ECM) that acts as an adhesive between the amnion/chorions and decidua tissues. It is present in cervicovaginal fluids during the first half of pregnancy and also as labor approaches

(Feinberg et al., 1991). Tests of fetal fibronectin levels have been used due to their higher sensitivity and negative predictive value when compared to many other clinical tests (Creasy et al., 2004).

Risk factors. Several risk factors are assessed to identify pregnancies at risk of PTB. Commonly obstetric history, socio-economic status, demographic characteristics (i.e. age and race), and premonitory symptoms have been used to predict women at risk; these, however, have not been reliable predictors (Creasy et al., 2004;Main et al., 1985). Risk factors for PTB include (Table 2-1a): multiple gestations, history of PTB, 2nd trimester bleeding, genitourinary tract infection, being African American, age less than 18 years, low body mass index (BMI), cigarette smoking, and frequent contractions (Goldenberg and Rouse, 1998;Meis et al., 1995;Meis et al., 1998). The risk factors with the largest OR are multiple gestations and a history of PTB, with ORs of 6 and 4, respectively.

Clinical markers. Several clinical markers have been used to assess risk including: cervical change (i.e. early cervical ripening), increasing contraction frequency, vaginal bleeding, and several biomarkers including fetal fibronectin, salivary-estriol, corticotrophin releasing hormone, interleukin 6 (IL-6), C-reactive protein, ferritin, and matrix-metalloproteinases among others (Crane et al., 1999;Creasy et al., 2004;Elovitz et al., 2001;Heine et al., 1999;Hobel, 2004;Holzman et al., 2001;Khan et al., 1999;Leitich et al., 1999;Lockwood et al., 1991;Main et al., 1985;Meyer et al., 1995;Ozer et al., 2005;Vogel et al., 2005). Among these early cervical ripening has been considered an indicator of a high-risk state for PTB. Cervical ripening induces a pronounced remodeling of the cervical ECM. The process itself is very similar to an inflammatory

response as it increases the expression of several cytokine proteins. However, cervical ripening is difficult to detect due to the subjective nature of the assessment. Biomarkers are typically only measured if a woman is suspected of being at risk of PTB; other clinical markers are commonly monitored over the course of pregnancy for most women, irregardless of PTB risk. These approaches have also proven to be inconsistent for predicting PTB (Copper et al., 1990;Lockwood and Dudenhausen, 1993).

Table 2-2. Summary of commonly used tocolytic treatments

Treatment	Common Targets	Usage
Magnesium sulfate	Myometrium contractions	Common
Prostaglandin synthesis inhibitors	Synthesis of prostaglandin G from arachidonic acid	Most common
Calcium antagonists	Voltage-gated ion channels (to reduce smooth muscle contractions)	Common
Beta-adrenergic agonists	Myosin light-chain kinase production	Less common

Treatment. PTB is commonly treated with corticosteroids, tocolytic agents, and antibiotics (Von Der Pool, 1998) Of these treatments, tocolytic therapies are most commonly used. Tocolytic approaches act by either inhibiting myometrial contractions or targeting prostaglandin synthesis. Tocolytic therapies often help to delay delivery so that corticosteroids can be administered, in efforts to enhance pulmonary maturity and reduce the severity of fetal respiratory distress syndrome therapies (deVeciana et al., 1995). There are four major established tocolytic pharmacological treatments for preterm labor: magnesium sulfate, prostaglandin synthesis inhibitors, calcium antagonists, and beta-adrenergic agonists (Creasy et al., 2004) (Table 2-2).

Magnesium sulfate has a depressant effect on the contraction of the myometrium, the precise mechanism by which the actions of magnesium occur, however, is not well understood. Magnesium sulfate in general does not have severe side effects, when administered in combination with other non-tocolytic drugs. Prostaglandin synthesis inhibitors (i.e. indomethacin) inhibit the cycloxygenase enzymes used in the conversion of arachidonic acid to prostaglandin G. Prostaglandin synthesis inhibitors can lead to several serious fetal side effects, including constriction of the ductus ateriosus, neonatal pulmonary hypertension, and oligohydramnios (Creasy et al., 2004). Finally, beta-adrenergic agonists (i.e. isoxsuprine, hexoprenaline, fenoterol, orciprenaline, ritodine, salbutamol, and terbutaline) increase beta2-adrenergic activity in the uterus by increasing the quantities of cyclic adenosine monophosphate, leading to a decrease in myosin light chain kinase production and inhibition of uterine contractions. The fetal effects of beta-adrenergic agonists include neonatal hypoglycemia, hypocalcemia, ileus, and hypotension (Brazy and Pupkin, 1979). Often, a physician uses a combination of tocolytic treatments, rather than just one, as guidelines for treatment vary. Tocolytic drugs, however, only provide short-term benefits and contribute to increased risks to both the mother and fetus that are not necessarily outweighed by their positive effects (deVeciana et al., 1995;Simhan and Caritis, 2007). In conclusion, there are no really good treatments for PTB.

Table 2-3. PTB rate (%) by race and Hispanic origin 1990-2005

Group	1990	1995	2000	2001	2002	2003	2004	2005
Total, All Races, Origins[1]								
Fewer than 32 weeks	1.69	1.61	1.58	1.57	1.57	-	-	-
32-36 weeks	8.01	8.21	8.54	8.81	8.87	-	-	-
Total, fewer than 37 weeks	9.7	9.82	10.12	10.38	10.44	12.30	12.50	12.70
Non-Hispanic Caucasians								
Fewer than 32 weeks	1.11	1.13	1.14	1.15	1.14	-	-	-
32-36 weeks	6.43	6.99	7.55	7.83	7.92	-	-	-
Total, fewer than 37 weeks	7.54	8.12	8.69	8.98	9.07	11.30	11.50	11.70
Non-Hispanic African Americans								
Fewer than 32 weeks	4.22	3.83	3.58	3.52	3.5	-	-	-
32-36 weeks	13.63	12.7	12.29	12.49	12.48	-	-	-
Total, fewer than 37 weeks	17.85	16.53	15.87	16.01	15.98	17.80	17.9	18.40
Hispanic[2]								
Fewer than 32 weeks	1.52	1.48	1.48	1.45	1.48	-	-	-
32-36 weeks	8.77	8.64	8.82	9.04	9.14	-	-	-
Total, fewer than 37 weeks	10.29	10.12	10.3	10.49	10.63	11.90	12.00	12.10

*Data from (Hamilton et al., 2006;Hamilton et al., 2007;Martin et al., 2002)
[1]Includes all races, including races not presented
[2]Includes all Hispanic racial identifications
*Rate is defined as a percentage

Epidemiology of Preterm Birth

Population disparity in prevalence rates

In addition to PTB's lifelong effects on the health of a child, there is a significant disparity in the occurrence of PTB among ethnic groups in the U.S. The prematurity rate amongst African Americans, for example, is ~18% compared to ~12% in Caucasians (Martin et al., 2003). Examining the trends of PTB over the last several years (Table 2-3) it is clear that rates have been on the rise over the last decade. When comparing the rates in African Americans and Caucasians they clearly have different trends; with the rate in African Americans having slowly declined and then increased over the course of the decade, while the rate in Caucasians has been gradually increasing. Interestingly, women of African descent born outside of the U.S. have a lower rate of PTB than women born

within the U.S. (Martin et al., 2002) and the rate of PTB at all educational levels in African American women is still higher than in their Caucasian counterparts (Kogan and Alexander, 1998).

The etiology of PTB seems to be different in African Americans relative to Caucasians. For example, African American women who experience PTB are more likely to have future PTBs relative to Caucasian women (Goldenberg et al., 1998; Creasy and Resnik 2004) and in African Americans early PTBs (less than 32 weeks gestation) occur more often with infection than they do in Caucasians (Creasy et al., 2004;Goldenberg and Andrews, 1996). Despite these differences the exact cause of the ~1.5 fold difference in rate between African Americans and Caucasians remains unexplained.

Preterm Birth Pathways

Mediators of several pathophysiological processes during pregnancy may genetically influence risk of PTB. Four pathogenic mechanisms have been well documented in published literature, from studies of both human and animal models (*in vivo* and *in vitro*), that can influence risk of PTB (Figure 2-1 from (Lockwood and Kuczynski, 2001)): 1) activation of maternal or fetal hypothalamic pituitary adrenal (HPA) axis; 2) decidual-chorioamniotic or systematic inflammation; 3) decidual hemorrhage (abruption) and 4) pathological distention of the uterus (Lockwood and Kuczynski, 2001). All mechanisms lead to a common terminal pathway that leads to the release of uterotonins, such as prostaglandins (lipids found in many vertebrate tissues that act as messengers in reproduction and inflammatory response). Increased uterine

contractility and cervical changes result due to activation and stimulation of the myometrium by increased concentrations of mediators including prostaglandins and oxytocin (Lockwood and Kuczynski, 2001).

Figure 2-1. Four pathophysiological pathways that incur increased risk of PTB

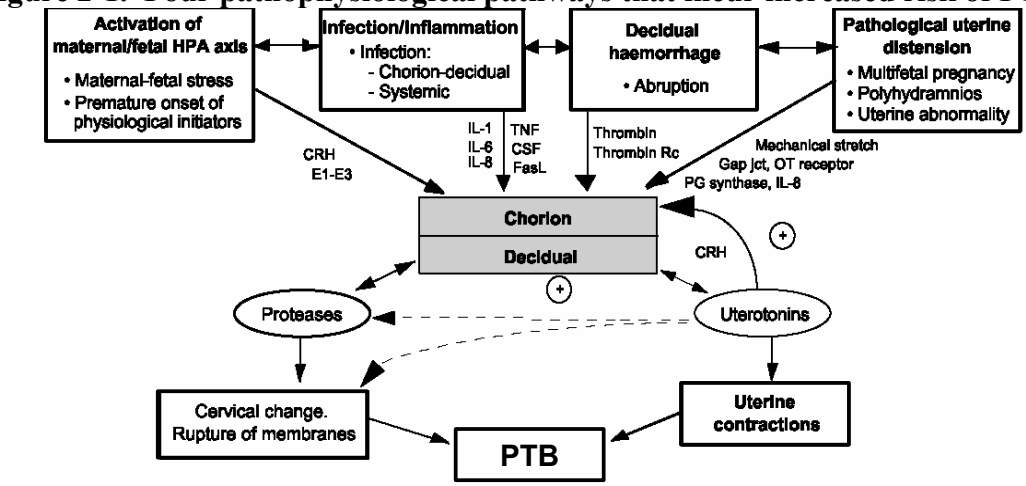


Figure 2-1. Four pathophysiological pathways influence risk of PTB. Adapted from, Lockwood et al (2001) *Paediatr. Perinat. Epidemiol*. 2, 560-567. The four proposed pathways are presented (activation of the maternal-fetal HPA axis, inflammation, decidual hemorrhage, and pathological uterine distension), as well as how they may potentially mediate risk of PTB.

Below are a few of ways that these four pathophysiological mechanisms can contribute to the activation of mediators leading to PTB:

1) **HPA-axis**: The HPA pathway is either stress-induced in the mother and/or precociously activated in the fetus. Increased stress levels increase fetal adrenal dehydroepiandrosterone sulfate (DHEAS) production, which is converted to estradiol and estrone. These products interact with the myometrium and cause an increase in gap junction formation, oxytocin receptor mRNA levels and prostaglandin activity, resulting in contractions (Meis, 2005).

2) **Infection and/or inflamatory response**: It is well established that endogenous host products mediate decidual-chorioamniotic and/or systematic inflammation (Belady et al., 1997;Goldenberg and Culhane, 2003). Inflammatory cytokine levels are often elevated in both AF and/or plasma of individuals experiencing preterm labor and early delivery suggesting that PTB may be a host inflammatory response (Draper et al., 1995;Fortunato et al., 2000;Vadillo-Ortega et al., 1990). Under this model PTB results from inflammatory response in fetal and/or maternal tissues generated either against an intrauterine infection or resulting from idiopathic causes. The activation of cytokines by infection induces prostaglandin production and the onset of myometrial contraction, labor, and birth. AF levels of a few cytokines associate with PTB including tumor necrosis factor alpha (TNF-α) (a trimeric protein encoded within the major histocompatibility complex) (Franchimont et al., 1998), and IL-6 (an important mediator in the host response to infection) (Simhan et al., 2003).

3) **Decidual hemorrhage**: Decidual hemorrhage (abruption) leads to a proteolytic cascade that can induce damage to the fetal membranes and promote PTB. Decidual hemorrhage is found in ~45% of patients with PTB (Salafia et al., 1995). It leads to local generation of increased levels of thrombin. Thrombin, with its pro-coagulant properties, binds to cellular receptors in the decidua and enhances the local protease and prostaglandin production. This may lead to degradation of laminin, fibronectin, and critical components of the decidua.

4) **Distension of the uterus**: Pathological distension of the uterus may be due to multiple gestations or polyhydramnios and is known to result in PTB. Multiple gestations or hydroaminos result in 3.3% of all pregnancies in the U.S. and contribute to

~15% of all PTBs (http://marchofdimes.com/peristats). Multiple gestations or hydroaminos lead to the stretching of the myometrium and fetal membranes, causing an increase in the synthesis of prostaglandins and COX2 expression, cervical maturation, membrane rupture, and uterine contractions (Leguizamon et al., 2001). Multiple gestations are becoming increasingly common as a result of artificial reproductive technologies which may explain a portion of the rate increase in PTB.

Genetic Studies on Preterm Birth

Genetic evidence

Despite the evidence supporting that a large portion of the risk can be explained by known non-genetic risk factors there is accumulating evidence supporting the hypothesis that PTB is influenced by genetic factors (Khoury et al., 1989;Treloar et al., 2000;Varner and Esplin, 2005). These include: 1) previous PTBs are associated with an increased risk of future PTBs; 2) an association has been seen between ethnicity/race and PTB; 3) mothers who were born preterm or have sisters who had a PTB have a higher risk of delivering PTB infants (Carr-Hill and Hall, 1985;Goldenberg and Andrews, 1996;Porter et al., 1997). In addition, twin studies estimate a heritability between 20 and 40% (Clausson et al., 2000;Treloar et al., 2000), this would suggest that despite known risk factors (Table 2-1) explaining a large portion of the risk for PTB, 20-40% of risk appears to be due to a genetic predisposition. These data, although not conclusive, strongly support a role for genetic variation in the etiology of PTB.

A factor that distinguishes genetic predisposition to PTB from many other phenotypes is that two distinct genomes can affect risk. The maternal-fetal interface has

long been thought to play an important role in PTB because both maternal and fetal tissues are involved in both maintenance of pregnancy and initiation of labor and delivery. For example, in infection driven PTB there is an immune response mounted by both mother and fetus in response to the presence of microorganisms at the decidual-membrane interface, initiated by microorganisms infecting the genital tract (Creasy et al., 2004). Another example is stress, maternal and/or fetal stress can initiate PTB by increasing corticotrophin releasing hormone and increasing levels of estrogen (Creasy et al., 2004). Further studies are necessary to determine which genome, mother or fetus, contributes most to risk of PTB.

Overview of established candidate genes

Several studies have been performed examining genes within all hypothesized PTB pathways for association with PTB. Table 2-4 provides a list of several candidate genes that have been associated with preterm birth (included are all clinical subtypes), and whether the association was found in maternal or fetal samples. Among these studies, infection and inflammatory response genes have been the focus of most studies due to reports documenting elevated cytokine levels in the AF/serum of PTB women. Below is an overview of a few established PTB candidate genes:

Table 2-4. List of candidate genes with positive associations

Gene	Gene Name	Population	Author Year
ACE	angiotensin I converting enzyme isoform 1	Maternal	(Valdez et al., 2004)
ADD1	adducin 1 (alpha) isoform a	Fetal	(Gibson et al., 2006)
ADRB2	adrenergic, beta-2, receptor, surface	Maternal	(Doh et al., 2004)
		Fetal	(Gibson et al., 2007)
		Maternal	(Landau et al., 2002)
DHFR	dihydrofolate reductase	Maternal	(Johnson et al., 2005)
FII	coagulation factor II precursor	Fetal	(Gopel et al., 1999)
FV	coagulation factor V precursor	Maternal	(Erhardt et al., 2000)
		Fetal	(Gopel et al., 1999)
FAS	tumor necrosis factor receptor superfamily,	Fetal	(Fuks et al., 2005)
IL-6	interleukin 6	Maternal	(Hartel et al., 2005)
		Maternal	(Simhan et al., 2003)
MBL2	soluble mannose-binding lectin precursor	Fetal	(Ameglio et al., 2007)
		Fetal	(Bodamer et al., 2006)
MTHFR	5,10-methylenetetrahydrofolate reductase	Fetal	(Chen et al., 2004b)
		Maternal	(Valdez et al., 2004)
MTRR	methionine synthase reductase isoform 2	Maternal	(Engel et al., 2006)
NOS2A	nitric oxide synthase 2A isoform 1	Fetal	(Gibson et al., 2007)
NOS3	nitric oxide synthase 3 (endothelial cell)	Fetal	(Gibson et al., 2007)
PON1	paraoxonase 1	Maternal	(Lawlor et al., 2006)
PPARG	peroxisome proliferative activated receptor	Fetal	(Meirhaeghe et al., 2007)
SERPINB2	serine (or cysteine) proteinase inhibitor, clade	Fetal	(Gibson et al., 2007)
SHMT1	serine hydroxymethyltransferase 1 (soluble)	Maternal	(Engel et al., 2006)
SLC23A2	solute carrier family 23	Maternal	(Erichsen et al., 2006)
THBD	thrombomodulin	Fetal	(Gibson et al., 2007)
TLR4	toll-like receptor 4	Fetal	(Lorenz et al., 2002)
TNF-α	tumor necrosis factor alpha	Fetal	(Aidoo et al., 2001)
		Fetal	(Menon et al., 2006c)
		Maternal	(Moore et al., 2004)
		Maternal	(Roberts et al., 1999)
sTNF-R1	soluble tumor necrosis factor receptor 1	Maternal	(Menon et al., 2006c)
sTNF-R2	soluble tumor necrosis factor receptor 2	Maternal	(Menon et al., 2006c)
VEGF	vascular endothelial growth factor isoform a	Maternal	(Papazoglou et al., 2004)
SERPINB2[1]	serine (or cysteine) proteinase inhibitor	Fetal	(Gibson et al., 2007)
TNF-α[1]	tumor necrosis factor alpha	Maternal	(Roberts et al., 1999)

[1]Results that trended positively toward association while the other studies were positively associated ($p < 0.05$)

Tumor necrosis factor alpha (TNF-α). The most extensively studied gene for association with PTB is TNF-α. Increasing evidence suggests that TNF-α levels vary greatly among individuals and is under genetic control (Mira et al., 1999). TNF-α is expressed by monocytes in response to microbial infection and in response to other

cytokines. TNF-α is a proinflammatory cytokine that is involved in remodeling of both the fetal membranes and the cervix by promoting the production of matrix metalloproteinases (MMPs). Studies reveal that women who have a history of PTB produce more TNF-α during pregnancy than women with a history of term delivery, suggesting a relationship between increased TNF-α concentrations and PTB (Amory et al., 2004a;Amory et al., 2004b).

One particular SNP in TNF-α at nucleotide G-308A has been extensively studied and has been associated with increased risk of PTB, chorioamnionitis, fetal morbidity, and pPROM (Louis et al., 1998). One of the first studies of G-308A observed associations of maternal G-308A genotype with pPROM in a small African American population (Roberts et al., 1999). This population contained a small PTB subset that did not associate with G-308A, consistent with studies by Dizon-Townson *et al.* (Dizon-Townson et al., 1997) but did trend towards positive. However, once G-308A was established as a potential candidate for PTB, a few small case-control studies examined this marker and found an association between maternal genotype and PTB in populations of mixed ancestry [Moore, 2004 5344 /id]. Studies have also examined G-308A with regard to fetal genotype. Aidoo *et al*. (2001) observed association of fetal G-308A genotype with PTB in a Western Kenyan population. This study was well powered, consisting of 1048 singleton fetal samples, all of the same ancestry. This association was successfully replicated in a Chinese population utilizing a trio study design (54 PTB infants and 79 control infants) (Chen et al., 2003).

A recent meta-analysis examining the associations of maternal G-308A suggests that observed associations at this marker may be spurious effects due to poor study design

(Menon et al., 2006a). Each of the studies described utilized a different ascertainment criteria, often consisting of pooled and/or mixed racial ancestry and for the most part suffered from poor sample sizes. Further studies will be necessary to determine the validity of the associations observed with fetal G-308A genotypes.

Interleukin 6 (IL-6). The second most commonly studied cytokine for association with PTB has been proinflammatory cytokine IL-6. IL-6 is an important mediator in the host response to infection. It activates acute phase response, stimulates T lymphocytes, and induces C reactive protein production (Simhan et al., 2003). IL-6 production influences human disease, especially those related to infection or inflammation. Its concentration in the AF and cervix is elevated in individuals experiencing PTB in comparison to other cytokine levels present in the AF (El-Bastawissi et al., 2000).

A (C→G) polymorphism at IL-6 C-174G decreases IL-6 promoter activity (Simhan et al., 2003). Homozygote (G/G) and heterozygotes (C/G) display normal production of IL-6, while (C/C) homozygotes display reduced concentrations. Simhan et al (2003) suggests that (C/C) homozygote individuals are less likely to have PTB. This study examined 51 PTB and 156 control births with an approximately two to one ratio of Caucasians to African Americans. This polymorphism also has a well documented ethnic disparity in both allelic and genotypic distributions between Caucasians and African Americans (Cox et al., 2001). Studies on IL-6 have also failed to replicate.

Coagulation factor V (FV). FV is not a candidate cytokine from the infection and inflammatory response pathway, but rather a candidate from the decidual hemorrhage pathway. FV is involved in blood coagulation, and is an integral component of the

compliment and coagulation cascade. Alteration in FV gene expression can result in both thrombotic and hemorrhagic diathesis. It was established as a candidate for PTB due to its potential involvement in decidual hemorrhage and due to documented associations between a FV marker at nucleotide G1691A, pregnancy complications, and venous thromboembolism (Camilleri et al., 2004;Morrison et al., 2002;Wuthrich, 2001).

FV is the only strong candidate for PTB identified by a high-throughput candidate gene screen (Hao et al., 2004). This study consisted of 300 maternal cases and 348 maternal controls, and three racial/ethnic groups (African American, Hispanic, and Caucasian). The majority of the samples (193 case and 260 controls) were African Americans. They identified a haplotype in FV, consisting of markers rs2072476, rs2072481, and rs2282743, that was statistically significant after a Bonferroni correction. The association was documented on pooled racial/ethnic populations; however, they noted that the association was stronger in African Americans and Hispanics than in Caucasians. The association in FV has not been replicated.

The utility of a high-throughput candidate gene association study

It is not justified at this time to posit any single factor as being critical in the initiation and maintenance of PTB. To date, a comprehensive candidate gene screen has not been adequately performed on PTB that encompasses all potential candidate pathways. Previous studies have also failed to address the potential genetic basis of the racial disparity in prevalence rates. Therefore, in the studies presented in the following chapters we responded to this deficiency by performing a comprehensive screen of established and potential PTB candidate genes from hypothesized PTB pathways and examining the relationships between several well-established PTB biomarkers. In

addition to this we also performed all analyses in both African Americans and Caucasians, given the established disparity in prevalence rates between the two groups, stratifying all analyses in these two groups.

A large-scale high-throughput candidate gene screen is the best genetic approach for identifying genetic factors that increase risk for this clinical phenotype and would greatly increase our ability to design a treatment for the prevention of PTB. Family studies have not been well documented in the literature due to the complex relationship between maternal and fetal genomes during pregnancy and due to the lack of adequate methodology to perform such a study (Wilcox et al., 1998). The following dissertation examines single locus associations with PTB due to individual effects of maternal and fetal genetic information, multilocus effects based on epistatic interactions, haplotype effects, and effects due to genetic interactions by mother and fetus. Combining all of these approaches and examining multiple SNPs in each of the candidate genes will be the first such study in this growing field of PTB research. This study has multiple lines of biological evidence to support the hypothesis that mediators in the hypothesized PTB pathways, defined by Lockwood et al (2001), are associated with PTB. This will also be the first study on PTB examining multiple SNPs at each candidate gene extending to a multilocus level and examining maternal and fetal contributions.

B. Hypothesis and Specific Aims

Hypothesis: Variations in multiple genes encoding proteins in four candidate pathophysiological pathways (activation of the maternal/fetal HPA axis, infection and/or inflammation, decidual hemorrhage, and pathological uterine distension) influence susceptibility to PTB. Additionally, there is an interactive effect between the genetic susceptibility of mother and fetus leading to PTB.

Specific aim I: Examine the role of maternal and fetal genetic control of biomarkers in PTB and term samples.

Amniotic fluid (AF) will be examined for protein concentrations of seven PTB candidate genes (interleukin (IL) -1, IL-6, IL-8, IL-10, tumor necrosis factor alpha (TNF-α), soluble TNF-receptor 1 (TNF-R1), and soluble TNF-receptor 2 (TNF-R2)). These cytokines will be examined for the correlated relationships both within African Americans and Caucasians. Finally, a well established candidate gene IL-6 will be examined for association between genotype and IL-6 AF protein expression**.**

Specific aim II: Select and genotype single nucleotide polymorphisms (SNPs) in multiple preterm birth (PTB) candidate genes for maternal and fetal PTB and term samples.

Approximately 130 genes were chosen based on physiological function and extensive search of the literature. Upon completion of the gene list, SNPs within each

gene were identified and selected based on several databases (i.e. HapMap, NCBI dbSNP, and Applied Biosystems) that are amenable to genotyping with an Illumina platform. A total of 1536 SNPs were selected for genotyping. All samples were genotyped for multiple SNPs in each candidate gene using the Illumina GoldenGate genotyping system.

Specific aim III: Test for association between candidate gene SNPs and PTB in maternal and fetal samples in a case-control population.

a. *Association analysis of the entire PTB dataset for African American and Caucasian maternal and fetal data separately by testing SNPs in the candidate genes for allele and genotype association with PTB.*
b. *Test for haplotype association with PTB in genes with multiple single locus associations in African American and Caucasian maternal and fetal data separately.*
c. *Perform multi-locus association analyses on the entire PTB dataset and within pathways for African American and Caucasian maternal and fetal data separately by testing for multi-locus genotype association with PTB.*

To better understand the genetic basis for the established differences in prevalence rates between African Americans and Caucasians, all analyses will be performed separately for African Americans and Caucasians. Single locus association analyses will be performed by using gestational age as a dichotomous variable. Tests for deviations from Hardy Weinberg equilibrium (HWE) will be performed within PTB and term births. Fisher's Exact test will be performed for tests of association with disease and

single locus alleles/genotypes. Tests for associations of multilocus genotypes with PTB will be performing using Multifactor Dimensionality Reduction (MDR) within the entire dataset and within pathway grouping of the data.

Specific aim IV: Test for association between mitochondrial DNA candidate gene SNPs and PTB in Caucasian maternal data.

a. *Association analysis of Caucasian maternal data by testing mitochondrial DNA SNPs for allele association with PTB.*
b. *Perform multivariate analyses on mitochondrial DNA SNPs adjusting for confounding demographic variables.*

All analyses will be performed for maternal Caucasian samples. χ^2 tests of association of disease with single locus alleles will be performed with gestational age as a dichotomous ourcome variable. Multilocus statistical interactions between markers will be evaluated with logistic regression. Results will be adjusted for effects of confounding variables.

Specific aim V: Test for interactions between genotypes of maternal and fetal samples.

Exploratory multilocus interactions between maternal and fetal samples will be examined by application of MDR to allow for the simultaneous analysis of maternal-fetal genotype combinations.

CHAPTER III

INTERLEUKIN (IL)-6 AND IL-6 RECEPTOR GENES AND SPONTANEOUS PRETERM BIRTH

Overview Chapter III Part A and B

PTB is a significant neonatal health problem that is more common in African Americans than in Caucasians. Part of this disparity is likely to result from the differing genetic architectures of Caucasians and African Americans. To begin assessing the role of these differences, patterns of genetic variation in two previously proposed candidate genes, in IL-6 (5 markers) and its receptor (IL-6R) (3 markers), were analyzed in mothers and fetuses in both Caucasians and African Americans. IL-6 concentrations in AF were determined in a subset of these pregnancies. Chapter III part A presents a small scale study examining these two candidate genes. No strong single marker effects were observed, however, all of the SNPs studied showed significant frequency differences between African Americans and Caucasians in at least one comparison; this was in significant excess of that expected from general population databases. Higher IL-6 concentrations associated with the IL-6 SNP -661 (rs1800797) in Caucasian preterm samples ($p= 6.00x10^{-3}$), and this result seems to be driven by MIAC, indicating a gene by infection interaction. Chapter III part B expands on the study performed in part A examining 8 markers in IL-6 and 22 markers in IL-6R focusing on associations between IL-6 AF and patterns of IL-6 genetic variation. Haplotype tests of association were performed by using a sliding window with a dichotomous (PTB/term) outcome and a continuous (IL-6 cytokine concentration (pg/ml)) outcome. In African Americans and

Caucasians significant results for both dichotomous and continuous outcomes converged on three regions: in IL-6 markers (rs1800797, rs1800796, and rs1800795); in IL-6R markers (rs4601580, rs4645618, rs6687726, and rs7549338); and IL-6R markers (rs4537545, rs4845374, and rs4845625). These findings indicate that, as a function of IL-6 genotype, Caucasian and African American women respond differently to infection with respect to their expression of IL-6. Our data support complex genetic control of levels of IL-6 in AF between Caucasians and African Americans.

(Chapter III part A adapted from Velez et. al. "Ethnic differences in interleukin 6 (IL-6) and IL-6 receptor genes in spontaneous preterm birth and effectson amniotic fluid protein leveles." *Ann. Hum. Genet*. 71: 586-600.)

A. Ethnic Differences in Interleukin (IL)-6 and IL-6 Receptor Genes in Spontaneous Preterm Birth and Effects on Amniotic Fluid Protein Levels

Introduction

PTB is a complex disease induced by several etiologic factors from potentially interacting pathophysiologic pathways (Lockwood and Kuczynski, 2001). These pathways culminate in a terminal biochemical pathway resulting in the activation of uterotonins, such as prostaglandins, that induce labor (Lockwood and Kuczynski, 2001). Of the different pathways that have been implicated in PTB, infection and the host inflammatory response to infection (e.g., cytokine and matrix metalloproteinase activation) are among the most commonly found precursors to PTB (Hillier et al., 1988;Romero et al., 1990;Romero et al., 2006;Romero and Mazor, 1988). Based on these data we hypothesize that the response to infection in some women increases their risk of

PTB compared to individuals of a different genetic constitution (Macones et al., 2004;Roberts et al., 1999).

In the US a ~2-fold difference in rates of PTB exists between African Americans and Caucasians. The rates of PTB in Caucasians and African Americans have converged; from 1989 to 1997 there was a 15.6% increase in the rate of PTB among Caucasians to 10.2% while African Americans experienced a 7.6% decrease to 17.5% over the same period (Demissie et al., 2001). This convergence of rates has continued with Caucasian PTB rate being 11.1% in 2002 and the African American rate being 17.5% (Martin et al., 2003). The causes of these changes in disparity are also not well understood, although it is possible that the disparity can be used to identify candidate risk factors that are significantly different between ethnic groups, thereby helping to define the underlying etiology of the phenotype.

One plausible candidate associated with infection that may affect the risk of PTB is IL-6. Increased concentration of this proinflammatory cytokine, in cervical and AF has been associated with MIAC in women delivering preterm (El-Bastawissi et al., 2000;Romero et al., 1990). Increased IL-6 concentration in cord plasma is also associated with fetal inflammatory response to maternal infection (Yoon et al., 2000). For these reasons IL-6 is considered a "marker" of high risk status for PTB (Romero et al., 1993). In addition, genetic association studies demonstrated that a single nucleotide polymorphism (SNP) in the promoter region of the IL-6 gene (C-174G) decreases promoter activity, and the (G/G) homozygote associates with increased risk of PTB (Hartel et al., 2004). IL-6 as well as its receptor (IL-6R), through which IL-6 mediates its

biological function, are therefore strong candidates for PTB and perhaps for the ethnic disparity in PTB.

The present association study was undertaken to better understand the patterns of variation of SNPs in the IL-6 and IL-6R genes. In this study, we compare the maternal and fetal allelic, genotypic, haplotypic patterns between African Americans and Caucasians and between cases (PTB) and controls (normal term deliveries) within African Americans and Caucasians. In addition, we assessed the effects of genetic variation on AF IL-6 concentration in cases and controls in both African Americans and Caucasians in efforts to understand ethnic disparity.

Materials and Methods

Study population

Caucasian and African American subjects were recruited at the Centennial Medical Center, Nashville, TN and Magee Women's Hospital, Pittsburgh, PA. Institutional Review Boards at Tri-Star Nashville, TN, Vanderbilt University, Nashville, TN and Magee Women's Hospital, Pittsburgh, PA approved this study. All subjects were recruited sequentially based on the availability of recruiters. All included pregnancies were singleton live births. Ethnicity was identified by self-report and a questionnaire that traces ethnicity back one generation from the mother and father. Individuals who had more than one racial group in their ancestry (one generation from mother and father) were excluded from the study. We recruited mothers between the ages of 18 and 40. Gestational age was determined by last menstrual period dating and corroborated by ultrasound dating. In our study, cases (PTB) were defined as presence of regular uterine

contractions at a minimum frequency of 2-contractions/10 minutes followed by delivery at <36 weeks gestation. We excluded subjects with multiple gestations, preeclampsia, preterm premature rupture of the membranes (pPROM), placental previa, fetal anomalies, gestational diabetes, poly- and oligohydramnios, and other complications such as surgeries during pregnancies. The controls consisted of women having normal labor and delivery at term (≥ 37 weeks) with no medical or obstetrical complications during pregnancy. A summary of the data recruitment and ascertainment for Centennial Women's Hospital data is described in detail on Figure 3-1. (Magee Women's hospital data recruitment is not presented, as it is not used in the studies presented in the following Chapters).

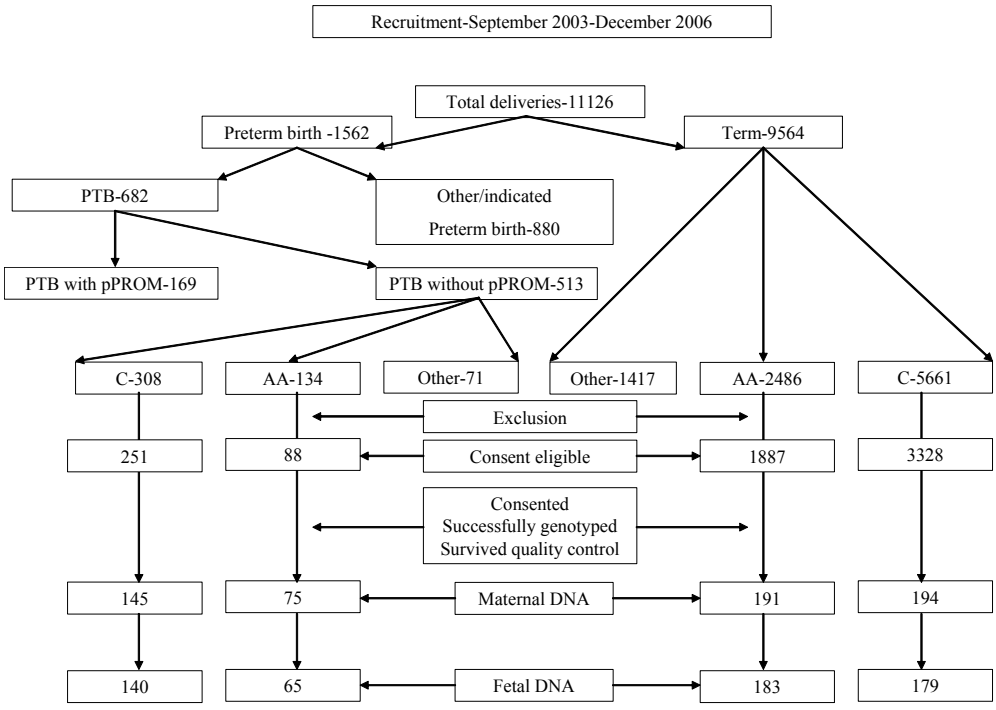


Figure 3-1. Data recruitment and ascertainment.
Data ascertainment procedure between September 2003 through December of 2006 is documented. The samples collected are outlined at each stage in ascertainment and are distributed by race. C refers to Caucasians and AA refers to African Americans.

Demographic and clinical characteristics

Our genetic study included a total of 496 birth events in Caucasians (149 cases and 347 controls) and 397 in African Americans (76 cases and 321 controls). Placental pathology was performed in all cases to document histologic chorioamnionitis and funisitis. The means and standard deviations of demographic and clinical characteristics for cases and controls are listed in Table 3-1. Significant differences between cases and controls were observed in Caucasians and African Americans for APGAR 1 (a method to assess a newborn infant, the score is a number calculated based on scoring heart rate, respiratory effort, muscle tone, skin color, and response to a catheter in the nostril 1 minute after birth (APGAR 1) and 5 minutes after birth (APGAR 5)), birth weight (kg) and gestational age at delivery (weeks) with $p<0.0001$.

Table 3-1. Clinical and demographic characteristics

Variable	Caucasian			African American		
	Mean		P-Value[1]	Mean		P-Value[1]
	Cases	Controls		Cases	Controls	
Age(yrs)	26.74 (6.43)	26.96 (5.90)	0.71	25.39 (5.91)	24.534 (5.572)	0.26
APGAR 1	7 (2.02)	8 (1.25)	<0.0001	6 (2.70)	8 (1.11)	<0.0001
Birth Weight (kg)	1.80 (0.70)	3.36 (0.50)	<0.0001	1.83 (0.79)	3.26 (0.52)	<0.0001
Gestation Age at Delivery(wks)	32.19 (3.16)	39.30 (1.21)	<0.0001	31.62 (4.29)	39.11 (1.13)	<0.0001
Gravidity	2.17 (1.29)	2.13 (1.45)	0.77	2.41 (1.46)	2.46 (1.60)	0.43

Means are reported with standard deviations reported in parentheses
[1]P-values compare cases to controls

DNA sampling and genotyping

DNA was isolated from maternal and fetal blood samples using the Autopure automated system (Gentra Systems (Minneapolis, MN)).

A total of eight SNPs were screened in IL-6 and IL-6R genes (5 in IL-6 and 3 in IL-6R). We used the reported minor allele frequencies of the SNPs (from National Institute of Health (NIH) dbSNPs database) and their relative distance on the gene as criteria for SNP selection. The rs numbers of SNPs from the NIH database, SNP locations, and relative distances are shown in Table 3-2. We did not use functional significance of SNPs as selection criteria with the exception of IL-6 -237 (rs1800795 also reported as -174) that was previously shown to associate with PTB (Simhan et al., 2003). Genotyping was done using the TaqMan assay on an ABI 7900 (Applied Biosystems of Foster City, CA).

Table 3-2. Positional information of SNPs

Gene	rs#	Location[1]	Position (bp)	Genic Region
IL-6 (Chromosome 7)	1880243	-7227	22532895	Promoter
	1800797	-661	22539461	Promoter
	1800796	-636	22539486	Promoter
	1800795	-237	22539885	Promoter
	1554606	1826	22541947	Intron
IL-6R (Chromosome 1)	6687726	22215	151213393	Intron
	4845622	33314	151224492	Intron
	4845623	37672	151228850	Intron

[1]Location refers to position from translational start site

Cytokine measurements

AF samples were collected during active labor (either preterm or term) by transvaginal amniocentesis before rupture of the membranes and delivery. Amniotic fluid samples were only collected in Nashville. Samples were collected before preterm or term vaginal deliveries by puncture of intact membrane using a 22 gauge needle prior to artificial rupture of the membranes. A few samples were also collected at the time of cesareans. AF was centrifuged immediately for 10 minutes at 2500 RPM to remove cellular and particulate matter. Aliquots of AF were stored at -70^{o}C until analysis.

IL-6 concentration in the AF was measured using multiple solid phase sandwich immunoassays utilizing antibody coated beads (Biosource International, Camarillo, CA) and analyzed with a Luminex^{TM} (Austin, TX). Standard curves were developed using duplicate samples of known quantities of recombinant proteins provided by the manufacturer. Sample concentrations were determined by relating the absorbance obtained to the standard curve by linear regression analysis. When necessary, samples were diluted in assay buffer provided by the manufacturer in addition to the doubling dilution required to perform the assay. The sensitivity of the assay was 1 pg/ml.

Both maternal genetic data and AF IL-6 concentrations were available on 54 Caucasian cases and 37 Caucasian controls, while both fetal genetic data and AF data were available from 57 Caucasian cases and 42 controls. In African Americans, 30 cases and 67 controls had both maternal genetic data and AF data, while 26 cases and 68 controls had both fetal genetic data and AF data.

MIAC was defined either by polymerase chain reaction (PCR) testing and/or clinical evidence. Presence of bacteria in the AF was detected by PCR amplification of

microbial 16s ribosomal DNA (TaqMan Assay, CA). Microbial cultures were not attempted since the PCR based detection of microbes is much more sensitive than traditional cultures (Gardella et al., 2004;Hitti et al., 1997). Microbial species identification using PCR was not performed in this study. Preterm cases with clinical evidence of MIAC were those individuals having three or more of the following criteria: abdominal tenderness, temperature > 38^{o}C, foul smelling vaginal discharge, an elevated C-reactive protein (CRP) or histologic chorioamnionitis.

Statistical methods

Single locus allele and genotype analysis. Shapiro-Walks tests of normality were performed to test for normality and as a result Mann-Whitney two sample rank sum tests were performed to compare demographic and clinical measurements between cases and controls. Single locus allele frequencies were analyzed, using the software Tools for Population Genetic Analysis, TFPGA, version 1.3 (available at http://www.marksgeneticsoftware.net/)(Raymond and Rousset, 1995). Genotype distributions at single sites were compared between ethnic groups using the program R X C at the same website, and are based on the Metropolitan algorithms. Single site Hardy-Weinberg Equilibrium (HWE) analyses were also performed using TFPGA (Raymond and Rousset, 1995). Statistical significance for the above was determined using Fisher's Exact Tests. All analyses were done on pooled Nashville and Pittsburgh samples because preliminary analyses showed that the two samples did not differ significantly within cases or within controls with regards to allele or genotype frequencies.

Haplotype analysis. Pairwise linkage disequilibrium (LD) was characterized and haplotype frequencies were calculated for Caucasians and African Americans, using Powermarker (Liu and Muse, 2005;Zaykin et al., 2002) and/or HaploView statistical software (Barrett et al., 2005). Results from the two software packages did not differ. Haplotype blocks were assigned using the algorithm created by Gabriel (Gabriel et al., 2002). Both Powermarker and HaploView use an EM algorithm to determine haplotype frequency distributions when phase is unknown. The Powermarker haplotype trend analysis was performed to test for haplotype frequency differences between groups. This approach can be applied to both quantitative traits and dichotomous traits. This analysis uses a regression approach to test haplotype-trait association. The test for association then uses an F test for a specialized additive model (Zaykin et al., 2002). This method was used to test for differences between African Americans and Caucasians and between phenotype classes within an ethnic group.

IL-6 AF concentration analysis. One and two-way analysis of variances (ANOVA) were used to examine AF IL-6 associations with IL-6 genotypes and pregnancy outcome and within Caucasians and African Americans. Both fetal and maternal genotypes were analyzed for association. STATA was used for ANOVA analyses (StataCorp, 2007). A log transformation was performed on IL-6 concentrations to normalize data because the Shapiro-Wilks test for normality demonstrated that cytokine concentrations deviated from normality. One-way ANOVA was performed with IL-6 markers to determine if the cytokine concentration differs with regards to an individual's genotype within a status groups. A two-way ANOVA was also performed with phenotype status and individual IL-6 markers as covariates, to determine if cytokine

concentration associates with status and individual markers. A two-way ANOVA was also used to assess association between marker and MIAC status within cases.

Correction for multiple testing

False discovery rate (FDR) was used to correct for multiple testing. FDR is used to measure global error, that is, the expected number of false rejections of the null hypothesis among the total number of rejections (Sabatti et al., 2003). FDR measures the proportion of false positives among all SNPs called significant. The FDR adjusted p-values are defined in a step-down fashion. The false discovery rate used was 0.20.

Results

Analysis of differential distribution IL-6 and IL-6R SNPs in African Americans and Caucasians

Significant differences between African Americans and Caucasians were observed at both the genotype and allele frequencies in IL-6 and IL-6R for maternal and fetal samples (Table 3-3). Of the 20 comparisons for allele frequency differences between Caucasians and African Americans in the IL-6 gene (maternal, fetal, cases and controls x 5 SNPs) , 13 were significant at the 0.05 level (3 in fetal cases, 3 in fetal controls, 3 in maternal cases, and 4 in maternal controls,) and 8 were significant with p values below 0.0001. Similarly, genotype frequency comparisons between the ethnic groups were different in 13 of 20 comparisons at the 0.05 level (3 in fetal cases, 3 in fetal controls, 3 in maternal cases, and 4 in maternal controls,) and 8 at the 0.0001 level. Comparisons of the IL-6R SNPs between racial groups were even more striking with 11 of 12 comparisons being significant at the 0.05 level for allele frequencies (2 in fetal cases, 3 in

fetal controls, and 3 each in maternal cases and controls). The only value not significant at the 0.05 level was fetal allele frequency at position 37672 in cases with a marginal p value ($p = 0.09$). The genotype comparisons for IL-6R were comparably different with 10 of 12 SNPs showing significant differences (2 in fetal cases, 3 in fetal controls, 2 in maternal cases, and 3 in maternal controls). It is clear from these data that most SNPs differed between ethnic groups within a phenotypic class in both maternal and fetal samples.

As might be expected from the patterns of single SNP differences in both fetal and maternal samples, haplotypes displayed strong differences in frequencies when comparing African Americans and Caucasians in both cases and controls. Highly significant differences ($p < 0.0001$) were observed in IL-6 and IL-6R haplotypes in all comparisons (Table 3-4).

Table 3-3. Single locus analysis of Caucasians and African Americans

a. Fetal

Marker	Allele	C		C		C		AA		AA		AA		C v AA Alleles		C v AA Genotypes	
		Allele Frequency		HWE P-Value[1]		Case-Control P-Value		Allele Frequency		HWE P-Value[1]		Case-Control P-Value		P-Value		P-Value	
		Case	Control	Case	Control	Allele[2]	Genotype[3]	Case	Control	Case	Control	Allele[2]	Genotype[3]	Case	Control	Case	Control
-7227	A	0.25	0.23	0.08	0.25	0.54	0.10	0.15	0.20	1.00	0.84	0.34	0.64	0.05	0.16	0.19	0.29
-661	A	0.38	0.36	0.07	0.01	0.64	0.91	0.09	0.11	0.35	0.21	0.6	0.86	<0.0001	<0.0001	<0.0001	<0.0001
-636	C	0.09	0.05	0.12	0.01	0.67	0.49	0.07	0.08	0.22	0.22	1.00	0.47	0.64	0.05	0.64	0.13
-237	C	0.40	0.37	0.01	0.01	0.37	0.61	0.08	0.11	0.29	0.22	0.47	0.54	<0.0001	<0.0001	<0.0001	<0.0001
1826	T	0.44	0.39	0.03	0.05	0.25	0.38	0.35	0.34	0.11	0.78	0.91	0.34	0.16	0.08	0.01	0.02
22215*	A	0.43	0.46	0.38	0.23	0.42	0.68	0.59	0.42	0.39	0.51	1.00	0.65	0.0075	<0.0001	0.04	<0.001
33314	C	0.43	0.39	0.52	0.89	0.40	0.53	0.18	0.14	0.62	0.18	0.19	0.15	<0.0001	<0.0001	<0.0001	<0.0001
37672*	G	0.47	0.42	0.18	0.49	0.34	0.15	0.57	0.44	1.00	0.82	0.91	1.00	0.09	<0.0001	0.18	<0.001

b. Maternal

Marker	Allele	C		C		C		AA		AA		AA		C v AA Alleles		C v AA Genotypes	
		Allele Frequency		HWE P-Value[1]		Case-Control P-Value		Allele Frequency		HWE P-Value[1]		Case-Control P-Value		P-Value		P-Value	
		Case	Control	Case	Control	Allele[2]	Genotype[3]	Case	Control	Case	Control	Allele[2]	Genotype[3]	Case	Control	Case	Control
-7227	A	0.21	0.22	0.13	0.59	0.75	0.51	0.20	0.15	1.00	0.67	0.30	0.34	0.78	0.002	0.61	0.01
-661	A	0.34	0.33	0.45	0.35	0.69	0.41	0.08	0.12	1.00	0.03	0.17	0.48	<0.0001	<0.0001	<0.0001	<0.0001
-636	C	0.07	0.06	1.00	0.33	0.67	0.49	0.09	0.08	1.00	0.14	0.86	0.55	0.56	0.13	0.39	0.31
-237	C	0.37	0.34	0.85	0.27	0.33	0.46	0.08	0.13	0.32	0.04	0.09	0.34	<0.0001	<0.0001	<0.0001	<0.0001
1826	T	0.43	0.38	0.38	0.15	0.17	0.13	0.29	0.32	0.12	0.68	0.46	0.48	0.008	0.02	0.01	0.04
22215*	A	0.43	0.44	0.21	0.22	0.89	0.88	0.54	0.42	0.61	0.72	0.42	0.64	0.05	<0.0001	0.12	<0.0001
33314	C	0.43	0.38	0.61	0.24	0.19	0.25	0.20	0.16	0.10	0.53	0.29	0.36	<0.0001	<0.0001	<0.0001	<0.0001
37672*	G	0.47	0.43	0.40	0.91	0.26	0.36	0.65	0.45	0.27	0.36	0.04	0.05	<0.0001	<0.0001	0.003	<0.001

[1] HWE-p-values testing for deviations from Hardy Weinberg Equilibrium
[2] Indicates comparisons between case and control allele frequencies.
[3] Indicates comparisons between case and control genotype frequencies
*Opposite minor alleles for these two markers in African Americans (AA) and Caucasians (C).

Table 3-4. Comparisons of Caucasian and African American haplotype frequencies

Fetal Sample		
Status	P-Value	
	IL-6	IL-6R
Case	<0.0001	<0.0001
Control	<0.0001	<0.0001
Maternal Sample		
Status	P-Values	
	IL-6	IL-6R
Case	<0.0001	<0.0001
Control	<0.0001	<0.0001

Case-control association analyses

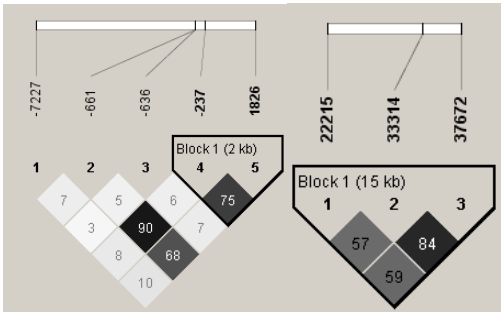
Deviations from HWE were observed in Caucasians at -661, -636 and -237 in fetal control samples (p= 0.01), 1826 in fetal controls (p= 0.05), -237 in fetal cases (p= 0.01), and 1826 in fetal cases (p= 0.03) in IL-6 (Tables 3-3a). Significant deviations from HWE were also observed in African Americans at -661 in maternal controls (p= 0.03), and -237 in maternal controls (p= 0.04; Table 3-3b). None of these p-values are corrected for multiple testing.

Single locus association analyses (allelic and genotypic) of Caucasian maternal and fetal samples found no significant associations between SNPs in IL-6 or in IL-6R and PTB (Table 3-3a and 3-3b) including the -237 SNP (commonly referred to as -174 from the transcription start site) that was previously shown to be associated with PTB. Allelic and genotypic analyses in African Americans found one significant association between the SNP at 37672 for IL-6R maternal samples (allele p= 0.04, genotype p= 0.05) with PTB (Table 3-3b). A marginally significant allele frequency difference between cases and controls was found at -237 for maternal samples (p= 0.09; Table 3-3b).

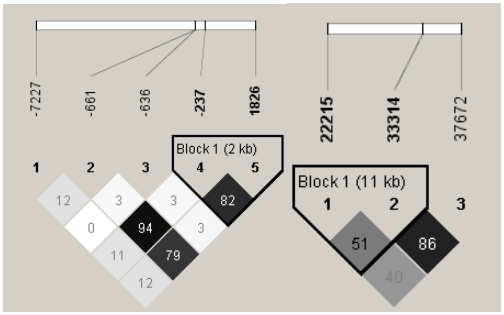
LD patterns were characterized for all populations examined, using r^2 as the metric (Figure 3-2). Caucasian controls had strong to moderate LD in both IL-6 and IL-6R in both fetal cases and controls. Fetal case and fetal control haplotype structure were similar to each other in Caucasians, as were Caucasian maternal case and Caucasian maternal control patterns (Figure 3-2a-d). IL-6 markers -237 and 1826 formed a block in all Caucasians samples. IL-6R markers 22215 and 33314 formed a block in all control samples as well as in Caucasian cases (Figure 3-2b, d, f, and h). As expected there was generally weaker LD present in African American samples as compared to Caucasians (Figure 3-2e-h). However, in African Americans LD appeared to be stronger in the control group as compared to the African Americans cases.

Haplotype regression analyses were also performed by treating PTB as a dichotomous variable (Table 3-5a and b). No significant associations were observed between haplotype frequencies and PTB in fetal or maternal samples in either ethnic group or either gene. Only haplotype frequencies present in 5.0% or more in at least one of the groups were included in the tables presented.

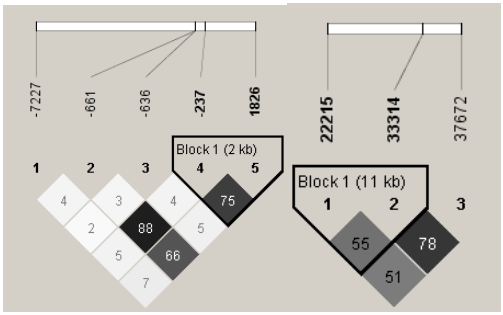
a. Caucasian fetal cases



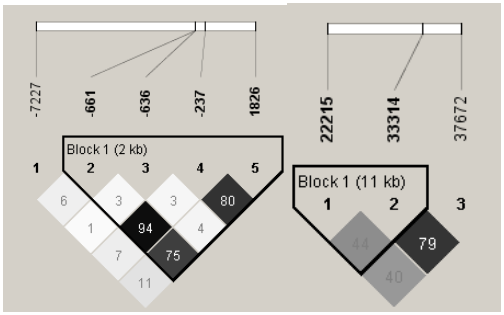
b. Caucasian fetal controls



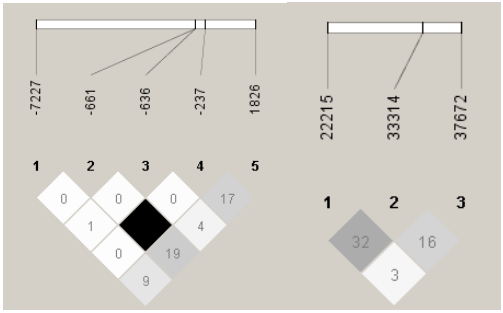
c. Caucasian maternal cases



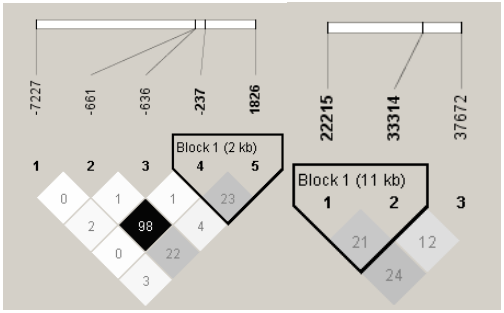
d. Caucasian maternal controls



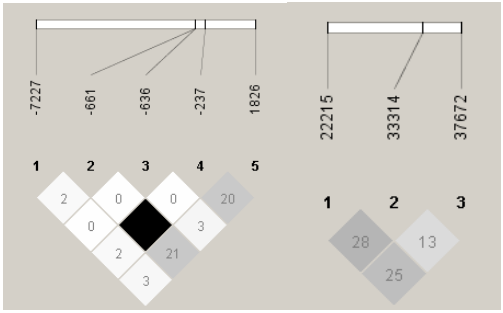
e. African American fetal cases



f. African American fetal controls



g. African American maternal cases



h. African American maternal controls

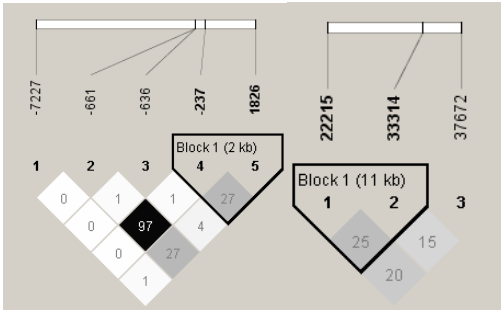


Figure 3-2. **Fetal and maternal interleukin 6 and interleukin 6 receptor linkage disequilibrium structure.**

Haplotype block structure and LD across IL-6 and IL-6R.The left hand side of each panel is IL-6 and the right and side is IL-6R. Numbers in the diamonds are r^2 values with darker shades of gray indicating higher values. Blocks are denoted as described in the text.

Table 3-5. Haplotype frequencies and trend regression results

a. IL-6

Fetal Samples

-7227	-661	-636	-237	1826	Frequency				P-Value	
					C		AA			
					Case	Control	Case	Control	C	AA
A	G	G	G	G	0.20	0.21	0.15	0.16	0.19	0.85
C	G	C	G	G	0.09	0.05	0.07	0.08		
C	G	G	G	G	0.26	0.34	0.43	0.42		
C	G	G	G	T	0.05	0.03	0.26	0.21		
C	A	G	C	T	0.33	0.34	0.09	0.10		

Maternal Samples

-7227	-661	-636	-237	1826	Frequency				P-Value	
					C		AA			
					Case	Control	Case	Control	C	AA
A	G	G	G	G	0.17	0.20	0.17	0.11	0.46	0.53
C	G	C	G	G	0.07	0.06	0.07	0.06		
C	G	G	G	G	0.33	0.35	0.47	0.48		
C	G	G	G	T	0.06	0.05	0.19	0.19		
C	A	G	C	T	0.30	0.31	0.07	0.11		

b. IL-6R

Fetal Samples

22215	33314	37672	Frequency				P-Value	
			C		AA			
			Case	Control	Case	Control	C	AA
A	A	A	0.42	0.43	0.32	0.38	0.75	0.09
A	A	G	0.02	0.03	0.27	0.2		
G	A	A	0.11	0.15	0.11	0.06		
G	A	G	0.03	<0.01	0.12	0.22		
G	C	G	0.43	0.38	0.18	0.14		

Maternal Samples

22215	33314	37672	Frequency				P-Value	
			C		AA			
			Case	Control	Case	Control	C	AA
A	A	A	0.41	0.40	0.32	0.38	0.52	0.39
G	A	A	0.12	0.16	0.04	0.08		
G	C	G	0.42	0.37	0.20	0.16		
A	A	G	0.03	0.03	0.22	0.20		
G	A	G	0.03	0.02	0.22	0.18		

*Caucasian-C; African American-AA

IL-6 amniotic fluid cytokine analysis

Overall, cytokine concentrations were higher in Caucasian cases than Caucasian controls, but this was not evident in African Americans (p values for all comparisons presented in Table 3-6). In Caucasian cases there is a significant association between maternal genotype at variant -661 and log IL-6 concentration (AA = 8.94±1.01 pg/ml, AG = 7.70±1.33 pg/ml, GG 8.92±1.39 pg/ml, $p = 6.00x10^{-3}$, Table 3-6 and Figure 3-3a). In comparison, there was no association in Caucasian controls (AA = 7.95± 0.98 pg/ml, AG = 7.37±1.27 pg/ml and GG = 7.54±1.13 pg/ml, p=0.59, Table 3-6 and Figure 3-3a). The two-way ANOVA also revealed a significant association for phenotype and -661 genotype in Caucasian mothers ($p = 8.00x10^{-3}$, Table 3-6b and Figure 3-3a). The African American samples did not show any differences in IL-6 concentration as a function of the genotype at -661 (Table 3-6c-d, Figure 3-3b). However, in African Americans there were only two significant associations between genotype and phenotype, and they were for -7227 for fetal genotype in control samples (p=0.04, Table 3-6c) and -636 maternal genotype in control samples (p=0.04; Table 3-6d).

Cases were also analyzed for the presence and absence of MIAC and for association between genotype and MIAC (Table 3-6a-d). In Caucasian cases a two-way ANOVA analysis for cytokine concentration, assessing maternal genotypes and MIAC, two SNPs were found to have significant associations, -661 ($p= 9.00x10^{-4}$) and -237 (p= 0.04) (Table 3-6b and Figure 3-3c) with MIAC. These markers were not statistically significantly associated with MIAC in African American cases. These results indicate a significant genotype by MIAC interaction in Caucasian cases. After performing an FDR correction, with a false discovery rate of 0.2, all statistically significant p values (original

cutoff p< 0.05) remain statistically significant, with the exception of the association of -237 and MIAC (p= 0.04) in Caucasian cases. In African Americans neither of the p-values remains significant after FDR correction.

Table 3-6. IL-6 cytokine concentration analysis

a.

Caucasian Fetal						
Marker	P-Value				Mean Cytokine Concentrations	
	Marker & Status[1]	Marker & MIAC[3]	Case (N=57)[2]	Control (N=42)[2]	Case (pg/ml)	Control (pg/ml)
-7227	0.95	0.86	0.68	0.55	8526.93	3294.96
-661	0.32	0.62	0.65	0.45		
-636	0.79	0.86	0.87	0.68		
-237	0.49	0.25	0.32	0.94		
1826	0.97	0.95	0.96	0.71		
Status[2]	$3.00x10^{-3}$					
MIAC	0.30					

b.

Caucasian Maternal						
Marker	P-Value				Mean Cytokine Concentrations	
	Marker & Status[1]	Marker & MIAC[3]	Case (N=54)[2]	Control (N=37)[2]	Case (pg/ml)	Control (pg/ml)
-7227	0.96	0.62	0.74	0.82	8557.30	3235.10
-661	$8.00x10^{-3}$	$9.00x10^{-4}$	$6.00x10^{-3}$	0.59		
-636	0.81	0.56	0.49	0.92		
-237	0.25	0.04	0.13	0.64		
1826	0.24	0.06	0.06	0.98		
Status[2]	0.01					
MIAC	0.41					

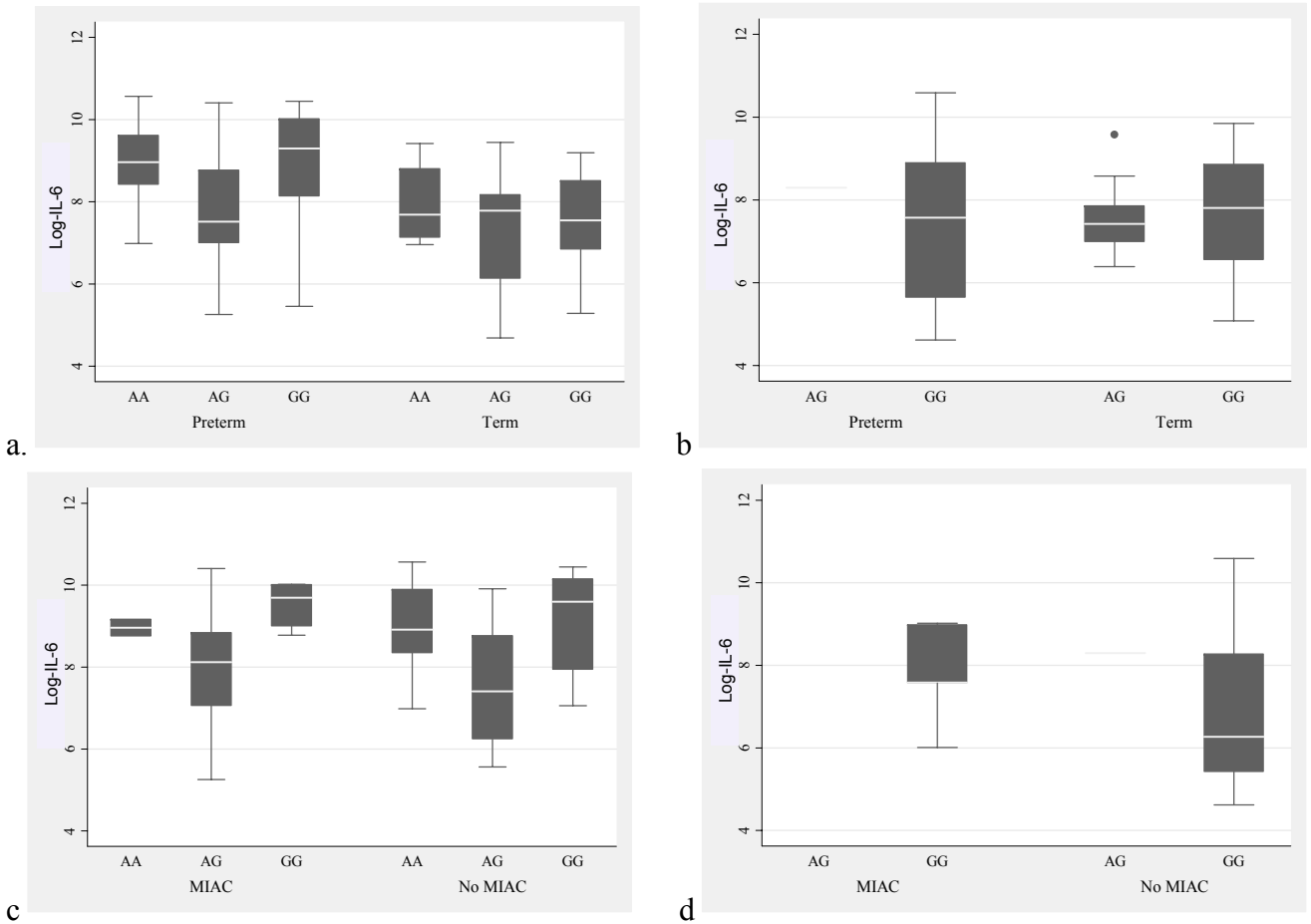
c.

African American Fetal						
Marker	P-Value				Mean Cytokine Concentrations	
	Marker & Status[1]	Marker & MIAC[3]	Case (N=26)[2]	Control (N=68)[2]	Case (pg/ml)	Control (pg/ml)
-7227	0.30	0.43	0.36	0.04	5055.61	4129.07
-661	0.59	0.67	0.46	0.40		
-636	0.74	0.40	0.60	0.35		
-237	0.59	0.70	0.47	0.40		
1826	0.55	0.56	0.72	0.71		
Status[2]	0.38					
MIAC	0.19					

d.

African American Maternal						
Marker	P-Value				Mean Cytokine Concentrations	
	Marker & Status[1]	Marker & MIAC[3]	Case (N=30)[2]	Control (N=67)[2]	Case (pg/ml)	Control (pg/ml)
-7227	0.79	0.41	0.62	0.50	4591.77	4256.86
-661	0.89	0.46	0.56	0.68		
-636	0.13	0.72	0.96	0.04		
-237	0.89	0.45	0.55	0.68		
1826	0.26	0.88	0.84	0.15		
Status[2]	0.27					
MIAC	0.34					

[1]Two way ANOVA with marker and status as covariates
[2]One way ANOVA
[3]Two way ANOVA with marker and infection as covariates within preterm cases only
*Caucasians-fetal with genotype and MIAC information = 15, without genotype and MIAC information = 39; Caucasians-maternal with genotype and MIAC information = 17, without genotype and MIAC information = 35; AA-fetal with genotype and MIAC information = 6, without genotype and MIAC information = 18; African American-maternal with genotype and MIAC information = 8, without genotype and MIAC information = 20



*Median cytokine concentrations indicated by a white line in box, lines indicates range of values

Figure 3-3. Median cytokine concentrations by genotype for IL-6 -661 in Caucasian and African American maternal samples. Median shown in the box plots for Caucasians by genotype (a) and African Americans by genotype (b). Caucasians by genotype and MIAC status is shown in (c) and African Americans by genotype and MIAC status is shown in (d).

Discussion

A case-control association study was performed to examine genetic variations in the IL-6 and IL-6R genes in efforts to explain ethnic disparity in the PTB rate between African Americans and Caucasians. Our data demonstrate that African Americans and Caucasians differ in allele, genotype, and haplotype frequencies. Additionally one IL-6R marker (37672) was found to statistically associate between cases and control within African American maternal samples. Our data also provide preliminary evidence that IL-6 AF concentration differs according to promoter variants in the IL-6 gene in Caucasian cases and that there is evidence for an interaction between maternal genotype and case-control and MIAC status in Caucasians, but not in African Americans.

With respect to patterns of genetic variation our results are very similar to previously published findings that found allele and genotype differences between Caucasians and African Americans for one of the SNPs we analyzed (rs1800795, which is typically referred to by its location to the transcriptional start site C-174G) (Cox et al., 2001;Hassan et al., 2003;Hoffmann et al., 2002). For example, in Cox *et al* the C allele frequency was reported as 0.35 in Caucasians and 0.09 in African Americans. We found that the C allele frequency was 0.37 in cases and 0.34 in control Caucasians and 0.08 in African American cases and 0.11 in African American controls (Cox et al., 2001), indicating that ethnicity correlates with differences in IL-6 gene variation. Our study, however, is more comprehensive than previously published ones in that we examined more markers and include both maternal and fetal samples. We, therefore, extended the findings to demonstrate that in both IL-6 and IL-6R there are many significant differences between African Americans and Caucasians. In addition, we identified

significant differences in haplotype frequencies in both maternal and fetal samples in both cases and controls between the two groups. Our data provide substantial evidence of ethnic differences in these genes.

To assess whether the differences we found are in line with overall genetic differences between Caucasians and African Americans, a χ^2 test comparing allele frequencies between African Americans and Caucasians was performed on all SNPs available on the Perlegen SNP genotype database (Hinds et al., 2005). Analyses of allele frequency differences between African Americans and Caucasians, using SNP genotype data available from the Perlegen database, demonstrate that 41.98% ($p < 0.05$) of SNPs differ between these groups. These p-values were not corrected for multiple testing. In our data, all of the SNPs examined demonstrated significant differences in allele frequencies between these two ethnic groups, and 7 of 8 did so at the genotype level, in at least one comparison. This is more than double the expected number of allele differences, indicating that the loci we analyzed differ between the ethnic groups more than expected from the genomic background.

The results from the single locus case-control analyses identified a single SNP at 37672 associated with PTB in IL-6R for both allelic and genotypic frequencies in African American mothers, although this result was not corrected for multiple comparisons. No significant haplotype associations were observed. These results are suggestive of the limitations of single locus associations in a complex phenotype such as PTB. It is possible that even though the genetic variants we studied affect PTB, they do so only in the context of other variants or other endogenous or exogenous risk factors. For example, we have already indicated that a multilocus interaction between TNF-α, IL-6 and IL-6R

genes are predictive of ~65% PTB in Caucasian women (Menon et al., 2006b). Other studies have documented that the effect of single promoter SNPs in IL-1β have different effects depending on what other SNPs are present elsewhere in the promoter region (Chen et al., 2006).

In addition to examining allele, genotype, and haplotype frequencies for association with PTB, we looked at the relationship between genotype, case status, and IL-6 AF concentration. We identified a significant association between IL-6 concentration and marker -661 in Caucasian maternal cases; this association is still significant after an FDR correction for multiple testing. This finding has several potential implications for the role of IL-6 on PTB. It suggests that AF IL-6 concentration is more closely associated with maternal than fetal genotype. However, the association with cytokine phenotype is not universal but differs by ethnicity and case status, indicating that other factors interact with these parameters to affect AF IL-6 concentration.

Cytokine analyses examining the effect of marker and MIAC status within case samples also revealed an association at IL-6 marker -661. This result remained statistically significant after an FDR correction, and is consistent with the interpretation that the IL-6 AF association between -661 and Caucasian cases is due primarily to MIAC status. Interestingly, a marginally significant association was also found at C-237G in Caucasians that was previously found to associate with PTB (Simhan et al., 2003). In our study, we found that C-237G and -661 are in strong LD. It is therefore likely that the previous association with C-237G may have actually been due to the effects of -661 or another SNP in very strong LD with it because -661, but not C-237G, significantly associates with AF concentrations.

The association of cytokine concentration with -661 genotype and MIAC and cytokine concentration differences seen between term and PTB individuals at -661 suggests that an interaction between maternal genotype and environment (MIAC) may in fact be important in PTB. These results have several implications for the understanding of the mechanism of PTB. First, our findings are supportive of a maternal genetic contribution to AF IL-6. This may be because maternal inflammatory cells (such as macrophages and NK cells) present in AF may function as major contributors of AF IL-6. Second, it suggests that IL-6 genotype may affect pregnancy outcome as indicated by previous studies (El-Bastawissi et al., 2000;Romero et al., 1990), but that the effects are not strong independent predictors of clinical phenotype, especially in the absence of an environmental factor such as MIAC. Instead, IL-6 concentration is more directly associated with SNP genotype as a product of gene by environment interaction, and that the genotype explains IL-6 concentration only as part of an interaction network with either other unstudied SNPs in this or other genes and/or environmental factors. Moreover, this association and interaction is limited to Caucasians making this finding a potentially important basis for ethnic disparity studies in PTB.

It is clear from our data that the distribution of genetic variation at the IL-6 and IL-6R genes differs significantly between Caucasians and African Americans. Given these findings and those of previous researchers it is reasonable to hypothesize a potential role for these genes in the disparity in PTB between Caucasians and African Americans. Such a conclusion is also supported by the relationship that we documented between genotype at an IL-6 promoter and AF IL-6 concentration in some but not all of our comparisons. Evidence for ethnic specific association with concentration provides even

more support for this hypothesis. However, although these data alone do not support a role for these genes in PTB, in conjunction with other genetic and physiological data the results strongly suggest a plausible functional role for them. In addition, our data provide a good baseline for understanding the patterns of variation in these populations that will be useful in future genetic epidemiological studies of this important phenotype.

B. Haplotypes in Interleukin-6 (IL-6) and Receptor (IL6-R) Spontaneous Preterm Birth (PTB) Candidate Genes Associate with Amniotic Fluid Protein Levels

Introduction

In a previous study, we examined a small subset of SNPs in IL-6 and IL-6R and found an association between AF IL-6 protein levels and a marker in the promoter region of IL-6, rs1800797 (G-661A), but only in Caucasian PTB (Velez et al., 2007a). The association between AF IL-6 concentrations and genotype at G-661A also differed between PTB with and without MIAC, showing evidence of a gene-environment interaction. This marker was also in LD with the previously described C-174G variant (described in Chapter II), indicating that a haplotype effect may account for the observed association with AF IL-6 concentrations. Support for this hypothesis was further provided by a report that haplotypes in the promoter region of IL-6, and not individual SNPs, control IL-6 gene expression (Terry et al., 2000). In the present study we analyzed tag SNPs in the IL-6 and IL-6R genes to better assess the relationship between AF IL-6 concentrations and genetic variation in the context of PTB. We focus on haplotype variation, as haplotype analyses may be able to better detect variants of etiological significance (Johnson et al., 2001). Given the disparity in PTB rates, we analyzed African American and Caucasian samples separately to provide insight into haplotype diversity and association across different ethnic groups. Specifically, we examined maternal and fetal DNA from PTB (cases) and term (controls) for single locus and haplotype associations within the IL-6 and IL-6R genes, using both dichotomous (case vs. control) and continuous outcome (AF IL-6 concentration).

Materials and Methods

Study population

African American and Caucasian subjects from Centennial Women's Hospital in Nashville, TN were used in the present study. Please refer to Chapter III part A for a complete description of subject recruitment and ascertainment.

Demographic and clinical characteristics

The total number of DNA samples included maternal and fetal samples from Caucasians (145 case and 194 control maternal; 140 case and 179 control fetal) and African Americans (75 case and 191 control maternal; 65 case and 183 control fetal).

DNA sampling and genotyping

DNA was isolated from maternal and fetal blood samples using the Autopure automated system (Gentra Systems (Minneapolis, MN)).

A total of 30 SNPs were screened in IL-6 and IL-6R genes (8 in IL-6 and 22 in IL-6R). We selected SNPs from a previously published publication (Velez et al., 2007a) (Chapter III part A) and additional SNPs based on their ability to tag surrounding variants from the HapMap (http://www.hapmap.org). The criteria for Caucasian tag SNPs were a minor allele frequency (MAF) of 0.07 and for African Americans a MAF of 0.20. For both we used an LD cutoff of $r^2 \geq 0.80$. The rs numbers of SNPs from the National Institute of Health (NIH) dbSNP database, chromosome, base pair (bp) position on chromosome, and SNP functions are shown in Table 3-7. We did not use functional significance of SNPs as selection criteria with the exception of IL-6 C-237G (rs1800795 also reported as C-174G) that was previously shown to associate with PTB and IL-6

expression (Simhan et al., 2003). Genotyping was performed using the Illumina GoldenGate genotyping system (Illumina, San Diego, CA)

Cytokine measurements

IL-6 concentrations in the AF were measured by multiplex assay (Biosource International, Camarillo, CA), using Luminex^{TM} (Austin, TX). The details of the assay procedure and details about the AF sample collection can be found in Chapter III part A. AF samples were measured from Caucasian (105 cases and 100 controls) and African American mothers (41 cases and 91 controls). Among these the following had genetic data: Caucasians (82 maternal cases and 40 maternal controls; 78 fetal cases and 36 fetal controls); African Americans (33 maternal cases and 57 maternal controls; 33 fetal cases and 57 fetal controls).

Table 3-7. SNP positions and functions

Gene	rs#	Position (bp)	Relative position[1]	Function
IL-6 (Chromosome 7)	rs1880243	22532895	-7227	Promoter
	rs12700386	22536249	-3873	Promoter
	rs1800797	22539461	-661	Promoter
	rs1800796	22539486	-636	Promoter
	rs1800795	22539885	-237	Promoter
	rs2069840	22541812	1690	Intron
	rs1554606	22541947	1825	Intron
	rs11766273	22548903	8781	3' UTR
IL-6R (Chromosome 1)	rs952146	151182001	-9178	Promoter
	rs1552481	151189426	-1753	Promoter
	rs6427641	151193559	2380	Intron
	rs11265610	151193857	2678	Intron
	rs1386821	151195122	3943	Intron
	rs4075015	151202269	11090	Intron
	rs4601580	151207490	16311	Intron
	rs4845618	151213088	21909	Intron
	rs6687726	151213393	22214	Intron
	rs7549338	151217453	26274	Intron
	rs4553185	151224028	32849	Intron
	rs4845622	151224492	33313	Intron
	rs4845623	151228850	37671	Intron
	rs4537545	151231952	40773	Intron
	rs4845625	151235140	43961	Intron
	rs4845374	151240020	48841	Intron
	rs11265618	151243165	51986	Intron
	rs10752641	151245115	53936	Intron
	rs4329505	151245493	54314	Intron
	rs2229238	151250969	59790	Exon
	rs4072391	151251953	60774	Exon
	rs7526293	151257282	66103	3' UTR

[1]Position relative to translation start site

Statistical analysis

Shapiro-Wilks tests of normality were performed on gestational age, gestational weight, APGAR 1 (1 minute after birth), APGAR 5 (5 minutes after birth), maternal age, and IL-6 cytokine concentration. All measurements deviated significantly from

normality with the exception of maternal age; as a result Mann-Whitney two-sample rank sum tests (Hollander and Wolfe, 1999) were used to test for statistical differences between medians of cases and controls of these variables; t-tests were used to test for statistical differences between case and control means for maternal age. STATA 9.0 statistical software (StataCorp, 2007) (College Station, TX) was used for all analyses.

Statistical tests for differences in single locus allele and genotype frequencies between ethnic groups and deviations from HWE were performed by the use of Powermarker statistical software (Liu and Muse, 2005;Zaykin et al., 2002). Statistical significance for these analyses was determined using Fishers Exact tests.

Pairwise LD was characterized and haplotype frequencies were calculated using Powermarker (Liu and Muse, 2005;Zaykin et al., 2002) and HaploView (Barrett et al., 2005) statistical software. Standard summary statistics D' and r^2 were calculated using HaploView (Devlin and Risch, 1995). Haplotype blocks were assigned using the D' confidence interval algorithm created by Gabriel *et al* (2002) (Gabriel et al., 2002). Both Powermarker and HaploView use an EM algorithm to determine haplotype frequency distributions when phase is unknown. The Powermarker haplotype trend analysis was performed with outcome as both dichotomous and continuous with 2, 3, and 4 marker sliding windows. A log transformed cytokine concentration was used for these analyses because the untransformed data were not normally distributed as assessed by a Shapiro-Wilks test and examination of distribution. This analysis is a regression approach to test haplotype-trait association. Haplotype trend analyses produce global p values for tests of haplotype associations (whether there is a different trend in haplotype frequency distributions between cases and controls for a dichotomous outcome or between different

strata of continuous outcomes). We performed analyses for cytokine concentrations initially on pooled cases and controls in order to narrow down the regions of interest for follow up with haplotype analyses, stratified by status. This was done under the presumption that pooled analysis would reveal most haplotype associations. For continuous outcomes, within each race p values were calculated within pooled cytokine-genotype data, then within each status group, and finally within cases with and without MIAC.

For case-control analyses the haplotypes with the most significant global associations were analyzed for haplotype specific effects. This included the calculation of OR for each haplotype, as well as determination of case and control haplotype frequencies. The highest frequency haplotype was used as the reference haplotype

Table 3-8. Clinical and demographic characteristics

Variable	African American			Caucasian		
	Case Mean(SD) or Median[IQR]	Control Mean(SD) or Median[IQR]	P-Value	Case Mean(SD) or Median[IQR]	Control Mean(SD) or Median[IQR]	P-Value
Gravidity (number of births)	2 [1-6]	2 [1-13]	0.96	2 [1-9]	2 [1-8]	0.02
Gestational Age (days)	242 [154-255]	273 [257-290]	<0.001	239 [166-255]	274 [257-296]	<0.001
Gestational Weight (grams)	2240 [462-3782]	3190 [1952-4517]	<0.001	2150 [370-3790]	3446 [2100-4661]	<0.001
APGAR 1	8 [1-9]	8 [3-9]	<0.001	8 [1-9]	8 [4-9]	<0.001
APGAR 5	9 [6-10]	9 [7-10]	<0.001	9 [1-9]	9 [7-10]	<0.001
Maternal Age (years)	25.32 (5.53)	25.22 (5.29)	0.88	27.33 (6.30)	28.39 (5.80)	0.10
Smoking (%)	36.67%	21.25%	0.08	31.77%	14.74%	<0.001

*Means are reported with standard deviations reported in parentheses and medians are reported with interquartile ranges in brackets

Results

Baseline characteristics

As expected by the case status, significant differences between cases and controls were observed in both Caucasians and African Americans for gestation age (days) (Caucasians $p < 0.001$; African Americans $p < 0.001$), birth weight (grams) (Caucasians $p < 0.001$; African Americans $p < 0.001$), APGAR 1 (1 minute after birth)(Caucasians $p < 0.001$; African Americans $p < 0.001$), APGAR 5 (5 minutes after birth)(Caucasians $p<0.001$; African Americans $p < 0.001$). Caucasians also had significant differences for gravidity ($p = 0.02$) and smoking ($p < 0.001$) (Table 3-8).

Single locus associations

Tests for HWE were performed for all SNPs in case and control samples separately in each population (Table 3-9). Among the 120 tests for deviations from HWE 8 deviated in cases ($p < 0.05$). In controls, of the 120 HWE tests, 17 deviated from HWE ($p < 0.05$), and one had a $p <0.01$. It is of note that for most situations where control $p < 0.05$ for HWE tests there is evidence that the inbreeding coefficients (F) were in opposite directions in cases and controls. For example, examining the 'F' in Caucasian maternal controls revealed that four of the markers that deviated from HWE in IL-6R showed that the directions of deviations from HWE was opposite to that of cases (rs4845622 - PTB F = -0.07 term F = 0.16; rs4845623 - PTB F = -0.06, term F = 0.18; rs5437545 – PTB F = 0.19, term F = -0.002; rs4329505 - PTB F = 0.04, term F = -0.13). These results suggest that the deviations are not likely due to genotyping error, as cases and controls were mixed in the same plates for genotyping.

In Caucasian maternal samples 4 marginally significant allelic associations were observed in IL6-R at rs7549338 (p = 0.04), rs4845625 (p = 0.04), rs11265618 (p = 0.05), and rs4072391 (p = 0.04) (Table 3-9c). No statistically significant single locus associations were observed in Caucasian fetal samples (Table 3-9c). African American maternal samples had one statistically significant association in IL-6 at rs2069840 (allelic association p = 0.03), but in IL-6R there were 8 SNPs with significant associations (either for allelic or genotypic association): rs4845618, rs6687726, rs4553185, rs4845622, rs4845623, rs4537545, and rs4845625 (Table 3-9d). African American fetal samples had two associated markers in IL-6 (rs1880243 and rs12700386), but none in IL-6R (Table 3-9d).

Linkage disequilibrium characterization

In Caucasian maternal and fetal DNA samples there is evidence for substantial LD in IL-6 in both cases and controls (Appendix Figure 1a-d). Two IL-6 SNPs in the 5' part of the gene had D' values of 1.0 in all Caucasian samples (rs1880243-rs12700386), although the pattern may be slightly different for other variants between cases and controls. As expected African American maternal and fetal samples showed less evidence of LD in IL-6. In fact, in the African American samples there was little evidence of LD in IL-6. Of particular note, the two most 5' markers that demonstrated complete LD in Caucasians showed no sign of LD in African Americans.

IL-6R had strong regions of LD in Caucasians; however, no blocks were identified according to the Gabriel *et al.* (2002) definition (Appendix Figure 2 a-d). The strongest regions of LD were in the 3' part of the gene between markers rs4845618 and rs7526293; weaker pairwise LD was observed in the 5' region between markers rs952146

and rs4075015. Again African American samples had weaker LD in comparison to Caucasians; however, several small blocks were identified (Appendix Figure 2 e-h). None of the blocks were consistent across status groups.

Table 3-9. Single locus association analysis of maternal and fetal samples

a. Caucasian IL-6

IL-6rs#	Allele	Maternal						Fetal					
		Allele Freq.		Case-Control Association P-value		[1]HWE P-Value		Allele Freq.		Case-Control Association P-value		[1]HWE P-Value	
		Control	Case	Allele	Genotype	Control	Case	Control	Case	Allele	Genotype	Control	Case
rs1880243	A	0.23	0.18	0.10	0.31	0.42	1.00	0.25	0.24	0.79	0.37	0.12	0.49
rs12700386	G	0.24	0.19	0.18	0.35	0.35	0.80	0.26	0.23	0.52	0.30	**0.05**	0.62
rs1800797	A	0.37	0.40	0.52	0.43	0.11	0.86	0.38	0.38	0.87	0.71	**0.01**	0.19
rs1800796	C	0.05	0.08	0.11	0.08	0.40	0.21	0.04	0.05	0.46	0.70	**0.02**	0.32
rs1800795	C	0.39	0.42	0.30	0.33	0.09	0.87	0.39	0.39	0.93	0.98	**0.04**	0.08
rs2069840	G	0.38	0.34	0.26	0.54	0.87	0.72	0.39	0.40	0.87	0.95	0.27	0.16
rs1554606	T	0.41	0.44	0.39	0.27	0.07	0.62	0.41	0.41	0.93	0.98	**0.04**	0.09
rs11766273	A	0.06	0.08	0.55	0.42	**0.03**	0.58	0.07	0.07	0.76	0.35	**0.01**	0.15

b. African American IL-6

IL-6 rs#	Allele	Maternal						Fetal					
		Allele Freq.		Case-Control Association P-value		[1]HWE P-Value		Allele Freq.		Case-Control Association P-value		[1]HWE P-Value	
		Control	Case	Allele	Genotype	Control	Case	Control	Case	Allele	Genotype	Control	Case
rs1880243	A	0.14	0.16	0.51	0.66	0.22	0.40	0.21	0.11	**0.01**	**0.03**	0.37	0.59
rs12700386	G	0.15	0.18	0.34	0.49	0.77	0.69	0.22	0.13	**0.03**	0.10	1.00	1.00
rs1800797	A	0.09	0.07	0.73	0.79	0.63	1.00	0.09	0.07	0.47	0.44	0.37	0.25
rs1800796	C	0.08	0.09	0.60	0.24	**0.01**	1.00	0.09	0.07	0.58	0.79	**0.04**	0.25
rs1800795	C	0.09	0.08	0.74	0.71	1.00	1.00	0.10	0.08	0.62	0.69	0.38	**0.05**
rs2069840	G	0.21	0.13	**0.03**	0.07	**0.02**	0.34	0.20	0.16	0.29	0.57	0.82	0.64
rs1554606	T	0.33	0.33	0.99	0.94	0.52	0.80	0.34	0.36	0.58	0.26	0.87	**0.03**
rs11766273	A	0.02	0.02	0.73	0.74	1.00	1.00	0.02	0.02	0.71	0.70	1.00	1.00

c. Caucasian IL-6R

IL-6R rs#	Allele	Maternal						Fetal					
		Allele Freq.		Case-Control Association P-value		[1]HWE P-Value		Allele Freq.		Case-Control Association P-Value		[1]HWE P-Value	
		Control	Case	Allele	Genotype	Control	Case	Control	Case	Allele	Genotype	Control	Case
rs952146	G	0.38	0.37	0.87	0.97	0.44	0.48	0.40	0.38	0.59	0.64	0.53	0.11
rs1552481	G	0.00	0.01	-	-	-	-	0.00	0.00	-	-	-	-
rs6427641	G	0.45	0.45	0.94	0.35	0.24	0.31	0.44	0.43	0.72	0.79	0.48	1.00
rs11265610	C	0.01	0.01	-	-	-	-	0.01	0.00	-	-	-	-
rs1386821	C	0.20	0.23	0.24	0.55	0.82	0.82	0.18	0.20	0.45	0.48	0.80	0.19
rs4075015	A	0.42	0.43	0.79	0.23	0.23	0.23	0.41	0.40	0.81	0.45	0.34	0.49
rs4601580	A	0.40	0.42	0.63	0.24	0.14	0.40	0.47	0.45	0.65	0.92	0.09	0.18
rs4845618	T	0.39	0.45	0.10	0.21	0.53	0.23	0.44	0.47	0.46	0.68	0.47	0.24
rs6687726	A	0.39	0.45	0.12	0.24	0.66	0.25	0.46	0.48	0.55	0.87	0.47	0.61
rs7549338	C	0.36	0.44	**0.04**	0.06	0.76	0.22	0.44	0.46	0.51	0.70	0.88	0.40
rs4553185	C	0.38	0.45	0.08	0.15	1.00	0.39	0.45	0.47	0.59	0.85	0.45	0.61
rs4845622	C	0.45	0.42	0.48	0.09	**0.03**	0.40	0.40	0.39	0.80	0.51	0.54	0.21
rs4845623	G	0.47	0.43	0.31	0.07	**0.02**	0.50	0.42	0.41	0.87	0.23	0.36	0.11
rs4537545	T	0.46	0.43	0.43	0.17	**0.01**	1.00	0.41	0.39	0.59	0.18	0.22	0.21
rs4845625	C	0.37	0.45	**0.04**	0.10	1.00	0.74	0.44	0.49	0.24	0.45	0.77	0.86
rs4845374	A	0.18	0.13	0.14	0.11	0.45	0.13	0.15	0.14	0.50	0.68	**2.29×10^{-3}**	0.27
rs11265618	T	0.19	0.13	**0.05**	0.12	0.82	0.48	0.17	0.14	0.38	0.61	**0.03**	0.14
rs10752641	G	0.21	0.26	0.08	0.24	0.66	1.00	0.26	0.26	0.92	0.11	0.70	**0.03**
rs4329505	C	0.17	0.13	0.12	0.06	**0.04**	0.53	0.14	0.14	1.00	0.97	0.76	0.47
rs2229238	T	0.17	0.23	0.06	0.13	0.62	1.00	0.21	0.22	0.69	0.40	0.50	0.22
rs4072391	T	0.17	0.23	**0.04**	0.13	0.61	0.83	0.21	0.22	0.66	0.24	0.36	0.31
rs7526293	T	0.19	0.24	0.09	0.21	0.63	0.82	0.22	0.22	0.86	0.39	0.65	0.32

d. African American IL-6R

IL-6R rs#	Allele	Maternal						Fetal					
		Allele Freq.		Case-Control Association P-value		[1]HWE P-Value		Allele Freq.		Case-Control Association P-value		[1]HWE P-Value	
		Control	Case	Allele	Genotype	Control	Case	Control	Case	Allele	Genotype	Control	Case
rs952146	G	0.40	0.41	0.84	0.71	0.29	0.10	0.38	0.43	0.37	0.52	0.88	0.33
rs1552481	G	0.19	0.18	1.00	0.79	0.48	0.71	0.17	0.18	0.79	0.92	1.00	1.00
rs6427641	A	0.31	0.39	0.07	0.14	1.00	0.63	0.34	0.38	0.41	0.55	0.31	0.80
rs11265610	C	0.28	0.25	0.55	0.24	0.47	0.13	0.25	0.23	0.67	0.55	0.43	0.15
rs1386821	C	0.10	0.12	0.53	0.10	0.70	0.06	0.11	0.10	0.75	0.64	0.12	0.51
rs4075015	A	0.11	0.14	0.34	0.12	0.48	0.14	0.12	0.12	1.00	0.64	0.28	0.58
rs4601580	T	0.43	0.41	0.78	0.42	0.31	0.23	0.41	0.42	0.92	0.60	0.28	**0.04**
rs4845618	G	0.46	0.58	**0.02**	**0.03**	0.19	0.23	0.44	0.51	0.19	0.30	0.56	0.13
rs6687726	G	0.38	0.48	**0.04**	0.09	1.00	0.49	0.38	0.40	0.58	0.78	0.64	0.30
rs7549338	C	0.38	0.26	**0.01**	**0.02**	0.54	0.13	0.40	0.35	0.35	0.27	0.47	0.18
rs4553185	T	0.39	0.52	**4.49×10^{-3}**	**0.01**	0.43	0.27	0.40	0.44	0.42	0.38	0.51	0.46
rs4845622	C	0.12	0.18	0.08	**0.02**	0.32	0.06	0.12	0.16	0.19	0.18	0.16	0.68
rs4845623	A	0.44	0.33	**0.02**	**0.02**	0.88	0.12	0.47	0.43	0.54	0.82	0.19	0.44
rs4537545	C	0.40	0.31	**0.05**	0.14	1.00	0.61	0.42	0.38	0.54	0.78	1.00	0.80
rs4845625	T	0.34	0.24	**0.03**	0.08	0.48	0.35	0.34	0.29	0.34	0.07	0.62	**0.02**
rs4845374	A	0.23	0.27	0.29	0.35	0.31	0.56	0.25	0.24	0.91	0.87	0.16	0.75
rs11265618	T	0.34	0.32	0.63	0.14	0.51	**0.03**	0.34	0.34	1.00	1.00	0.74	1.00
rs10752641	G	0.36	0.30	0.21	0.39	0.26	1.00	0.37	0.40	0.56	0.79	0.87	0.60
rs4329505	C	0.34	0.35	0.75	0.91	0.11	0.31	0.33	0.30	0.59	0.25	0.40	**0.04**
rs2229238	T	0.22	0.18	0.23	0.25	0.15	0.69	0.22	0.22	0.90	0.22	0.39	0.07
rs4072391	T	0.33	0.31	0.70	0.32	0.20	0.41	0.31	0.28	0.51	0.10	0.29	0.07
rs7526293	T	0.42	0.42	1.00	0.88	0.77	0.33	0.42	0.41	0.84	0.98	0.75	0.63

[1]HWE-Hardy-Weinberg equilibrium deviation p-values

*All significant p values ($p < 0.05$) are labeled in bold

Table 3-10. IL-6 sliding window haplotype analysis results

a. Dichotomous outcome

Population	Markers	Haplotype	Frequencies		OR	95% CI	p	Global p
			Cases	Controls				
Caucasian Maternal	rs12700386-rs1800797-rs1800796-rs1800795	C-G-G-G	0.35	0.34	1.00	-	-	0.05
		C-A-G-C	0.35	0.35	0.97	0.66-1.42	0.87	
		G-G-G-G	0.15	0.22	0.65	0.33-1.29	0.07	
		C-G-C-G	0.08	0.05	1.57	0.77-3.22	0.18	
		G-A-G-C	0.05	0.02	2.42	0.92-6.85	0.06	
Caucasians Fetal	rs2069840-rs1554606-rs11766273	G-G-G	0.40	0.39	1.00	-	-	0.01
		C-T-G	0.34	0.33	1.01	0.69-1.48	0.97	
		C-G-G	0.18	0.20	0.86	0.56-1.38	0.53	
		C-T-A	0.07	0.06	1.19	0.58-2.43	0.60	
African American Maternal	rs1800795-rs2069840	G-C	0.79	0.72	1.00	-	-	0.05
		G-G	0.13	0.18	0.67	0.37-1.17	0.14	
		C-C	0.08	0.07	1.01	0.45-2.17	0.97	
African American Fetal	rs1880243-rs12700386	C-C	0.80	0.72	1.00	-	-	0.02
		C-G	0.09	0.07	1.15	0.51-2.46	0.70	
		A-G	0.05	0.15	0.32	0.12-0.73	4.00×10^{-3}	
		A-C	0.07	0.05	1.24	0.48-3.03	0.60	

b. Cytokine concentration outcome

Population	Markers	Haplotype	Mean Log(pg/ml)		Cytokine p (Global(Case/control))[1]
			Case	Control	
Caucasian Maternal	rs12700386-rs1800797	G-G	3.58	3.24	0.04 (0.36/0.34)
		G-A	3.47	2.90	
		C-G	3.41	3.23	
		C-A	3.44	3.31	
Caucasian Fetal	rs1554606-rs11766273	G-G	3.60	3.28	0.05 (0.14/0.51)
		T-A	3.23	3.06	
		T-G	3.39	3.26	
African American Maternal	rs1554606-rs11766273	G-G	3.46	3.39	0.05 (0.68/0.03)
		T-A	3.37	2.91	
		T-G	3.29	3.13	
African American Fetal	rs1800797-rs1800796	G-G	3.40	3.19	0.02 (0.58/0.02)
		G-C	3.30	2.95	
		A-G	2.99	3.34	
African American Fetal	rs1800796-rs1800795	G-G	3.40	3.19	0.02 (0.58/0.16)
		G-C	2.99	3.34	
		C-G	3.30	2.95	

[1]Cytokine p values are presented by global p for the pooled data, followed by p for analyses within cases and within controls

Table 3-11. IL-6R sliding window haplotype analysis results

a. Dichotomous outcome

Population	Markers	Haplotype	Frequencies		OR	95% CI	p	Global p
			Cases	Controls				
Caucasian Maternal	rs4845625-rs4845374	T-T	0.41	0.45	1.00	-	-	0.02
		C-T	0.45	0.37	1.34	0.95-1.89	0.08	
		T-A	0.13	0.18	0.80	0.49-1.29	0.34	
African American Maternal	rs4075015-rs4601580-rs4845618	T-A-T	0.30	0.31	1.00	-	-	0.01
		T-T-G	0.26	0.25	1.07	0.63-1.82	0.79	
		T-A-G	0.20	0.18	1.12	0.62-1.99	0.70	
		T-T-T	0.10	0.15	0.68	0.32-1.36	0.24	
		A-A-G	0.07	<0.001	-	-	$1.00x10^{-3}$	
		A-A-T	0.02	0.08	0.17	0.02-0.71	0.01	
		A-T-G	0.06	0.02	2.89	1.00-9.12	0.05	
African American Fetal	rs4845625-rs4845374	C-T	0.47	0.42	1.00	-	-	0.04
		T-T	0.29	0.34	0.76	0.46-1.25	0.25	
		C-A	0.24	0.25	0.86	0.51-1.46	0.56	

b. Continuous cytokine concentration outcome

Population	Markers	Haplotype	Mean Log(pg/ml)		Cytokine p (Global(Case/control))[1]
			Case	Control	
Caucasian Fetal	rs4601580-rs4845618	A-G	3.67	3.22	1.60×10^{-3} (0.04/0.63)
		A-T	3.50	3.19	
		T-G	3.43	3.24	
		T-T	3.30	3.43	
African American Maternal	rs4601580-rs4845618-rs6687726-rs7549338	A-G-G-G	3.77	3.13	2.30×10^{-3}(0.03/0.05)
		A-G-A-G	3.45	3.04	
		A-T-G-G	3.59	3.29	
		A-T-G-C	3.50	3.20	
		T-G-A-G	3.10	3.30	
		T-T-G-G	2.65	2.86	
		T-T-G-C	3.84	3.49	
African American Fetal	rs4537545-rs4845625	T-T	3.40	3.23	0.02 (0.33/0.08)
		T-C	3.27	3.17	
		C-C	3.55	2.91	

[1]Cytokine p values are presented by global p for the pooled data, followed by p for analyses within cases and within controls

a. IL 6

Table 3-12. MIAC analyses of haplotypes identified with continuous outcome

Population	Markers	Haplotype	Mean Log (pg/ml)		P-Values	
			MIAC[1]	No MIAC[2]	MIAC[1]	No MIAC[2]
Caucasian Maternal	rs12700386-rs1800797	G-G	3.60	3.55	0.64	0.29
		C-G	3.69	3.35		
		C-A	3.53	3.39		
		G-A	-	3.48		
Caucasian Fetal	rs1554606-rs11766273	G-G	3.82	3.51	0.11	0.44
		T-A	3.44	3.11		
		T-G	3.51	3.38		
African American Maternal	rs1554606-rs11766273	T-G	3.65	3.39	0.29	0.84
		G-A	3.44	3.24		
African American Fetal	rs1800797-rs1800796	A-G	-	2.99	$<1.00x10^{-3}$	0.72
		G-C	-	3.30		
		G-G	3.50	3.35		
African American Fetal	rs1800796-rs1800795	C-G	-	3.30	$<1.00x10^{-3}$	0.72
		G-C	-	2.99		
		G-G	3.50	3.35		

b. IL6-R

Population	Markers	Haplotype	Mean Log (pg/ml)		P-Values	
			MIAC[1]	No MIAC[2]	MIAC[1]	No MIAC[2]
Caucasian Fetal	rs4601580-rs4845618	A-G	3.56	3.72	0.66	0.16
		A-T	3.69	3.45		
		T-G	3.59	3.40		
		T-T	3.75	3.15		
African American Maternal	rs4601580-rs4845618-rs6687726-rs7549338	A-G-G-G	4.16	3.63	0.69	0.17
		A-T-G-G	3.30	3.50		
		T-G-A-G	3.85	3.44		
		T-T-G-C	3.49	3.50		
		A-G-A-G	3.07	3.11		
		A-T-G-C	3.29	2.54		
African American Fetal	rs4537545-rs4845625	T-T	3.45	3.43	0.06	0.31
		T-C	3.61	3.19		
		C-C	-	3.00		

[1]MIAC-8 African American maternal, 7 African American fetal, 22 Caucasian maternal, 19 Caucasian fetal
[2]No MIAC- 25 African American maternal, 26 African American fetal, 59 Caucasian maternal, 57 Caucasian fetal

IL-6 haplotype and case-control status

Sliding window haplotype analyses in Caucasian maternal samples found that haplotypes defined by SNPs rs12700386-rs1800797-rs1800796-rs1800795 associated with pregnancy outcome (global p = 0.05). The G-G-G-G (OR 0.65 [CI 0.33-1.29], p = 0.07) and the G-A-G-C haplotype (OR 2.42 [CI 0.92-6.85], p = 0.06) showed the strongest associations (Table 3-10a). Caucasian fetal sample analyses found association with haplotypes defined by rs2069840-rs1554606-rs11766273 (global p = 0.01) (Table 3-10a), but no single haplotype showed significant association.

In African American maternal samples an association for a two SNP haplotype defined by rs1800795-rs2069840 (global p = 0.05) was found, but no single haplotype significantly associated (Table 3-10a). African American fetal analyses found an association with SNPs rs1880243-rs12700386 (global p = 0.02). This result is due to the A-G (OR = 0.32 [CI 0.12-0.73], p = 4.00×10^{-3}) haplotype that confers a significant protective effect relative to the most common haplotype, C-C.

IL-6 haplotype and IL-6 concentration

In Caucasian maternal samples haplotypes defined by rs12700386-rs1800797 associated with AF log transformed cytokine concentrations in pooled analyses of cases and controls (p = 0.04) but showed no significant association when stratified by status (cases p = 0.36; controls p = 0.34) (Table 3-10b). Of interest is the observation that rs12700386-1800797 haplotypes have apparently different patterns of association depending on clinical status. The G-A haplotype associated with a higher concentration in cases [3.47 log(pg/ml)] compared to controls [2.90 log(pg/ml)]). These two markers also have different LD structure in cases and controls with D' = 0.75 in controls and D' =

0.36 in cases (Appendix Figure 1a and b). Caucasian fetal sample haplotype analyses found that rs1554606-rs11766273 associated with IL-6 concentrations (pooled p = 0.05) however this is probably due to the fact that the haplotype associations trended in the same direction in cases and controls even though they were not significant (cases p = 0.14; controls p = 0.51) (Table 3-10b).

Haplotype analyses in African Americans revealed associations in both maternal and fetal samples with two site haplotypes (Table 3-10b). In maternal samples rs1554606-rs11766273 haplotypes associated with IL-6 concentration in controls (p=0.03) but not in cases (p = 0.68). Haplotypes for rs1554606-rs11766273 had different trends in IL-6 concentrations in cases and controls, with T-A having the lowest IL-6 concentration in controls [2.91 log(pg/ml)]) but T-G having the lowest concentration in cases [3.29 log(pg/ml)]). These two SNPs are in high LD in both cases and controls (D' = 1.00), but rs11766273 has low MAF (0.02) in both cases and controls in African Americans. For African American fetal sample haplotype analyses the rs1800797-rs1800796 haplotype associated with IL-6 concentrations (pooled cases and controls p = 0.02; cases p = 0.58; controls p = 0.02) as did rs1800796-rs1800795 (pooled cases and controls p = 0.02; cases p = 0.58; controls p = 0.16) (Table 4b). In both the rs1800797-rs1800796 and rs1800796-rs1800795 IL-6 haplotype mean concentrations trended in opposite directions with respect to case status. For example, for rs1800796-rs1800795 the G-G haplotype has the lowest concentration in cases [2.99 log(pg/ml)]) but the highest in term [3.34 log(pg/ml)]). The three marker haplotype including rs1800797-rs1800796-rs1800795 did not associate with AF IL-6.

IL-6R haplotype analyses and case-control status

In Caucasian maternal samples IL-6R haplotype rs4845625-rs4845374 showed significant association with PTB (global p = 0.02) (Table 3-11a). The C-T haplotype had the strongest association (OR = 1.34 [CI = 0.95-1.89 [, p = 0.08). No fetal haplotype associated with pregnancy outcome.

In African American mothers a three SNP haplotype associated with cases, rs4075015-rs4601580-rs4845618 (global p = 0.01) (Table 3-10a). In addition to there being global significance, three individual haplotypes associated: A-A-G (frequency 0.07 in cases, undetected in controls, OR uncalculated, p < 0.001), A-A-T (OR = 0.17 [CI 0.02-0.71]; p = 0.01), and A-T-G (OR = 2.89 [CI 1.00-9.12], p = 0.05). These differences appear to be driven by rs4845618 as indicated in the single SNP association (Table 3-8c). In African American fetal samples rs4845625-rs4845374 haplotypes associated with PTB (global p = 0.04), but no haplotype associated individually.

IL-6R haplotype and IL-6 concentration

Haplotype analyses of IL-6R found several haplotypes associated with IL-6 concentration, although none for Caucasian maternal haplotypes. A two SNP Caucasian fetal haplotype at rs460158-rs4845618 associated significantly with IL-6 concentration (pooled p = 1.60×10^{-3}; case p = 0.04; control p = 0.63) (Table 3-11b). As in some situations above trends differed between cases and controls; in cases the A-G haplotype had higher IL-6 concentration (3.67 log(pg/ml)) compared to controls (3.22 log (pg/ml)).

In African American maternal samples haplotypes defined by rs4601580-4845618-rs6687726-rs7549338 significantly associated with IL-6 concentration (pooled p = 2.30×10^{-3}; case p = 0.03; control p = 0.05). The analysis provided evidence for

similar trends in mean cytokine concentrations between cases and controls (Table 3-11b). In African American fetal sample haplotypes there was an association with rs4537545-rs4845625 and IL-6 concentration (pooled p = 0.02; case p = 0.33; control p = 0.08). Case and control mean cytokine concentrations trended in different directions.

Haplotype-MIAC analyses

Cases stratified by MIAC were examined for IL-6 and IL-6R haplotype association (Table 3-12) in IL-6 and IL-6R. Only African American fetal samples showed evidence for differences in haplotype associations between cases with and without MIAC for IL-6 rs1800797-rs1800796 (MIAC p = < $1.00x10^{-3}$; no MIAC p = 0.72) and rs1800796-rs1800795 (MIAC p = < $1.00x10^{-3}$; no MIAC p = 0.72) and in IL-6R rs4537545-rs4845625 (MIAC p = 0.06; no MIAC p = 0.31).

Discussion

This study examined 30 polymorphisms in IL-6 and IL-6R for association with PTB as an extension of our previous study of many fewer SNPs in these genes with the intent of assessing haplotype association (Velez et al., 2007a) (Chapter III part A). This analysis was performed with the intention of identifying common regions in both IL-6 and IL-6R across African Americans and Caucasians that influences risk of PTB. Several statistically significant haplotypes were found in both Caucasians and African Americans with both dichotomous and continuous cytokine outcome; however, three regions in the two genes appear to be shared in several of the comparisons (Figure3- 4a and b).

In the IL-6 gene one small region in the promoter region including markers rs1800797-rs1800796-rs1800795 showed significance in both Caucasian and African

American maternal and African American fetal samples; two significant associations were observed with IL-6 concentration and two with PTB. In our previous report one SNP in this region showed evidence for association with IL-6 concentration (rs1800797) (Velez et al., 2007a). African American fetal samples had two independent overlapping haplotypes and Caucasian maternal samples had one overlapping haplotype in this region. In Caucasians SNPs in this region are in very strong LD (pairwise D' 0.96-1.00). In African American maternal and fetal samples only two of these markers have strong pairwise LD (rs1800797-rs1800795, D' = 1.00), with stronger overall LD in this region in controls relative to cases in both maternal and fetal samples. This indicates that there may be an effect caused by either an ungenotyped variant in the region or a haplotype effect.

Our results are in agreement with a previously published report of IL-6 promoter haplotypes (Terry et al., 2000). This study examined rs1800797, rs1800796 and rs1800795 in Caucasians in which haplotypes within the IL-6 promoter region (including rs1800797, rs1800796, rs1800795 and -373A_nT_n) were shown to influence IL-6 transcription. The results demonstrated that upon IL-1 stimulation IL-6 expression is greatest with the G-G-G (rs1800797-rs1800796-rs1800795) haplotype but this same haplotype shows lowest IL-6 concentration in the absence of stimulation (Terry et al., 2000). In our study this haplotype did not associate with IL-6 concentration; however, in Caucasian maternal samples markers rs12700386-rs1800797-rs1800796-rs1800795 associated with PTB status with the G-G-G-G haplotype being protective. African American fetal data also associated with IL-6 concentration with two overlapping haplotypes (rs1800797-rs1800796; rs1800796-rs1800795). The fact that African

American fetal samples have two haplotypes rather than one may suggest a recombination event split a single functional haplotype into two independent functional haplotypes. Across the analyses, three markers in this haplotype (rs1800797-rs1800796-rs1800795) were found to have the most overlapping associations, suggesting that a variant tagged by this haplotype may be playing an important regulatory in AF IL-6 production in cases, regardless of ethnicity.

In IL-6R two regions (rs4601580-rs4845618-rs6687726 and rs4537545-rs4845625-rs4845374) associated with a phenotype in more than one analysis. Both regions contain intronic and exonic markers (Figure 3-4b). These two regions had the strongest overall haplotype associations and also had more associations in African Americans than Caucasians, especially with IL-6 concentration as outcome. Of note, two haplotypes in maternal Caucasian samples (rs4845618-rs6687726-rs7549338, $p = 0.06$ and rs4537545-rs4845625, $p = 0.04$ both for PTB) and one in fetal Caucasian samples (rs453745-rs4845625, $p = 0.02$ IL-6) that are not represented in the figures also had some evidence for association in these two regions. Although these were not the most strongly associating haplotypes, they were significant or of borderline significance, and these data further support the likelihood that these regions of the IL-6R gene affect phenotype.

Comparing the strength of the results in the IL-6 gene to that of IL-6R indicates that IL-6R rather than IL-6 has more influence on AF IL-6 concentration. This may be because these SNPs are in close proximity and in LD with functional/coding parts of the gene. For example, markers rs4845618 and rs6687726 are in close proximity to several exons and rs4537545-rs4845625-rs4845374 have exons between them. The CEU LD structure from the HapMap for IL-6R markers shows that both of the interesting regions

in IL-6R are all in LD and in close proximity to several coding variants. The part of the IL-6R gene from rs4537545-rs4845625-rs4845374 was found to associate with increased IL-6 concentration (Rafiq et al., 2007). This study found that a variant in IL-6R (rs4537545) associated with both IL-6 and IL-6R serum concentrations; this variant did not associate with inflammatory status. It was observed that these markers associate with several metabolic traits, e.g. diabetes, and marginally associates with cardio-vascular disease and asthma. Another recent study found that IL-6 concentrations are more associated with IL-6R variants than IL-6 gene variants (Reich et al., 2007). This study identified a variant (rs8192284 – between markers rs4845374 and rs11265618) in Caucasians and African Americans that associated with IL-6 concentrations; this marker is located downstream of in the 3' most region in IL-6R that we identified (Reich et al., 2007).

In our haplotype/AF analyses opposite trends in AF IL-6 concentrations were observed between cases and controls for some of the same haplotypes. This indicates that the same haplotypes can affect IL-6 concentrations as a function of status and ethnicity. Haplotype trend regression analyses within cases and controls for AF concentrations revealed different trends in AF haplotype levels between ethnic groups in cases with MIAC further supporting this conclusion.

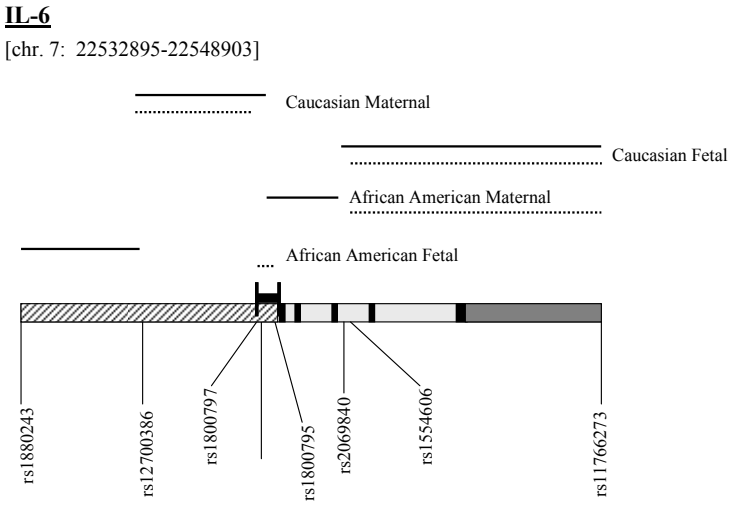
We were underpowered to detect an association with our MIAC subset; however, we observed association between IL-6R haplotypes, MIAC, and IL-6 concentrations in African Ameircan cases with MIAC, but not in those without MIAC. No differences were seen for cases with and without MIAC for any of the haplotypes associated with cytokine concentration as outcome. This is indicative of environmental influence (i.e.

infection) on IL-6 production in African American fetal samples but not in the others. However, in previous studies we observed that at the single SNP level MIAC plays a strong regulatory role on IL-6 production within Caucasian maternal samples (Velez et al., 2007a) especially at rs1800797. Interestingly, rs1800797 overlaps with the haplotypes found to associate with MIAC in African American fetal data. These data further support the hypothesis that a gene-environment interaction is occurring in PTB in both African American fetal and Caucasian maternal genes; the mechanism, however, is unclear but is consistent with a strong regulatory role by the markers within the region including rs1800797 in the IL-6 gene and this result is consistent across ethnic groups.

Previous results and our present study indicate that both IL-6 and IL-6R haplotype variation contribute to variations in AF IL-6 concentrations in both ethnic groups. Different maternal and fetal associations were observed in the two groups. Our dichotomous and continuous analyses were consistent for variants in IL-6 and/or elevated IL-6 levels being a risk factor for PTB. Results also suggest that some haplotype effects may be common across African Americans and Caucasians, although our analyses were designed to increase chances to detect commonalities. Our data also support the hypothesis that the regulation by genetic variation is dependent on a variety of other factors such as infection, but others may be operating, including patterns of variation at other genes related to inflammatory processes. Demonstrating the role of other factors will however require further investigation. Although we identified interesting statistical associations, the interpretation of their biological relevance is currently unclear. Functional studies will need to be performed to assess the biological implications of these associations and these gene regions in IL-6 and IL-6R. In conclusion, haplotype

association analyses revealed three regions in IL-6 and IL-6R that associate with AF IL-6 in PTB.

a.



b.

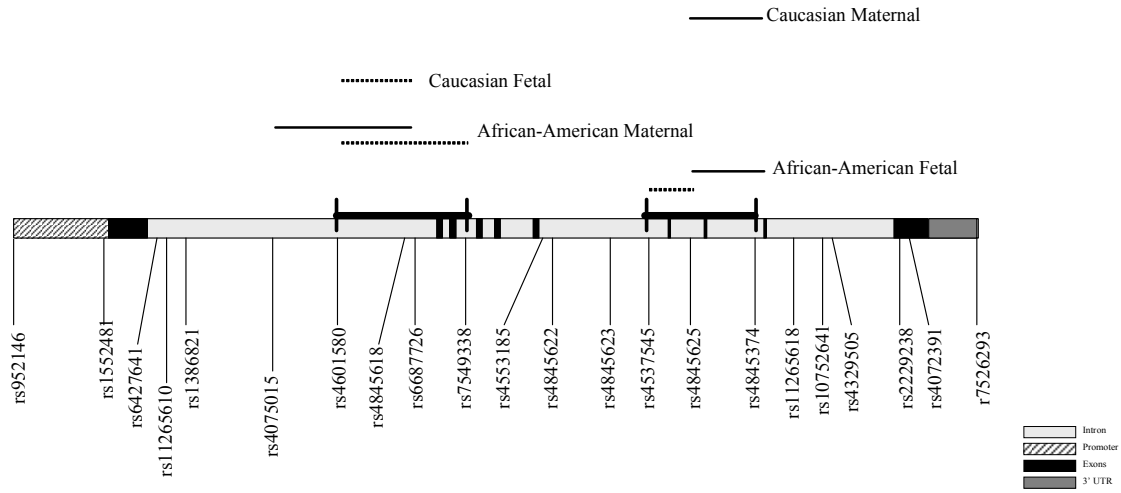


Figure 3-4. Haplotype summary

Dark lines with bounded vertical lines indicate haplotypes that were associated with dichotomous outcome and dashed lines indicate haplotype that were associated with continuous outcome, dark bounded lines on the x-axis indicate regions of overlapping haplotype associations. Introns are labeled with light grey, promoter with dashed lines, exons with solid black, and 3'UTR with dark grey. a) Results for haplotype analyses for IL-6; b) results for haplotype analyses for IL-6R.

CHATPER IV

A LARGE SCALE HIGH-THROUGHPUT CANDIDATE GENE ASSOCIATION STUDY OF PRETERM BIRTH IN CAUCASIANS AND AFRICAN-AMERICANS

Overview Chapter IV Part A and B

A large-scale candidate gene association study was performed examining 1536 SNPs in 130 novel and previously tested candidate genes from established PTB pathways. Maternal and fetal DNA from 370 Caucasian birth-events (172 cases and 198 controls) and 279 African American birth-events (82 cases and 197 controls) were examined. Single locus, haplotype, and multi-locus association analyses were performed on maternal and fetal data separately. These analyses will be presented in two sections, Chapter IV part A presents the results from our Caucasian samples and part B presents the results from our African American samples. Multilocus analyses were also performed and an overview of those analyses is presented in Chapter V. In the Caucasian maternal data the strongest associations were found in genes in the complement-coagulation pathway related to decidual hemorrhage in PTB. The single strongest effect was observed in tissue plasminogen activator (tPA) marker rs879293 with a significant allelic ($p = 2.00x10^{-3}$) and genotypic association ($p=2.0x10^{-6}$) with PTB. The best model had an OR of 2.80 [CI 1.77 - 4.44] for GG v AG&AA. The single strongest effect in fetal samples was observed in the inflammatory pathway at rs17121510 in the interleukin 10 receptor antagonist (IL-10RA) gene for allele ($p = 0.01$) and genotype ($p = 3.34x10^{-4}$). The best model had an OR 1.92 [CI 1.15-3.19] ($p = 2.00x10^{-3}$) for the additive model. Finally, multi-locus analyses in the compliment and coagulation pathway were performed

and revealed a significant interaction between a marker in coagulation factor V (FV) (rs2187952) and coagulation factor VII (FVII) (rs3211719) ($p < 10^{-3}$). In both African American maternal and fetal data the strongest associations were found in genes related to infection and inflammatory response pathways. The strongest associations were in interleukin 15 (IL-15) (rs10833, allele $p = 2.91 \times 10^{-4}$, genotype $p = 2.00 \times 10^{-3}$) in maternal data and interleukin 2 receptor beta (IL-2RB) (rs84460, allele $p = 1.37 \times 10^{-4}$, genotype $p = 6.29 \times 10^{-4}$) in fetal data. The ORs for the best models in maternal and fetal data were additive (rs10833 OR = 0.54[CI 0.37-0.78], $p = 1.0 \times 10^{-3}$; rs84460 OR = 2.36[CI 1.49-3.73], $p < 1.0 \times 10^{-3}$). Finally, multi-locus analyses identified tumor necrosis factor alpha (TNF-α) (rs1800683) and interleukin 10 (IL-10) (rs3024498) ($p = 0.03$) from the T cell receptor signaling pathway from fetal samples as being a potentially interesting interaction. These results support a strong role for complement and coagulation pathways in Caucasian maternal data, infection and inflammation pathways genes in African American PTB, and suggest that there may be different maternal and fetal genetic risks for PTB in African Americans.

A. A Large Scale High-throughput Preterm Birth Candidate Gene Association Study in Caucasians

Introduction

It has become well established that PTB has a genetic component (Adams et al., 2000;Carlini et al., 2002;Goldenberg and Andrews, 1996;Varner and Esplin, 2005). Supporting this is the fact that among the known risk factors of PTB the most significant is a family history of PTB (Adams et al., 2000;Carlini et al., 2002;Goldenberg and

Andrews, 1996;Varner and Esplin, 2005). Twin studies also estimate ranges for the heritability of PTB between 20-40%, suggesting that as many as 40% of PTB can be attributed to genetic causes (Clausson et al., 2000;Treloar et al., 2000). Associations have also been seen between ethnicity/race and PTB (Goldenberg and Andrews, 1996;Zhang and Savitz, 1992). Although, these data are not conclusive they do suggest that genetic variation may increase or decrease PTB susceptibility.

Data from previously published studies in both *in vivo* and *in vitro* human and animal models suggests that four primary pathogenic pathways either independently or through interactions lead to PTB (Lockwood and Kuczynski, 2001). The four proposed pathways are: 1) activation of maternal or fetal hypothalamic-pituitary-adrenal (HPA) axis; 2) decidual-chorioamniotic or systematic inflammation; 3) decidual hemorrhage (abruption) and 4) pathological distention of the uterus (Lockwood and Kuczynski, 2001). All pathways culminate in a common terminal pathway that causes the release of uterotonins, such as prostaglandins, leading to labor, and eventually PTB (Lockwood and Kuczynski, 2001) (Figure 2-1).

Given the complex and poorly understood etiology underlying PTB and the increasing trends of PTB rates in Caucasians, we chose to perform a large-scale PTB candidate gene association study focusing on a Caucasian population. We examined 1536 SNPs and given the complex exchange of genetic information at the maternal-fetal unit during pregnancy, we analyzed maternal and fetal data from 130 new and previously assayed candidate genes in the hypothesized pathways. Single locus, haplotype, and multi-locus tests of association (PTB – case; term birth – control) were performed, genes

were grouped into pathways according to biological process and analyzed according to these pathways.

Materials and Methods

Study population

Caucasian subjects from Centennial Women's Hospital in Nashville, TN were used in the present study. Please refer to Chapter III part A for a complete description of subject recruitment and ascertainment.

Demographic and clinical characteristics

The present study included Caucasian maternal and fetal samples (145 case and 194 control maternal; 140 case and 179 control fetal). Placental pathology was performed in all cases to document histologic chorioamnionitis and funisitis. Smoking status was determined from a questionnaire.

DNA sampling and genotyping

DNA was isolated from maternal and fetal blood samples using the Autopure automated system (Gentra Systems (Minneapolis, MN)). A total of 1536 SNPs were screened in 130 candidate genes (4 genes were within 5000 kb of candidate genes and were analyzed as members of the established candidate). We chose SNPs based on their ability to tag surrounding variants in the CEPH and Yoruba population of the HapMap database (http://www.hapmap.org) using a minimum minor allele frequency (MAF) of 0.07 in CEPH and 0.20 in Yoruba and r^2 0.80, given that our genotyping was performed in conjunction with an African American sample. 1432 markers remained after removing monomorphic markers (markers with MAF < 0.05) and markers that were not genotyped

successfully from Illumina (please refer to Appendix Tables 1 and 2 for a complete list of genes and SNPs). As a quality control step all matched mother-baby pairs were analyzed for Mendelian inconsistency, all samples with over 1% of their markers having inconsistencies were removed from analyses (24 samples dropped due to Mendelian inconsistencies). We also included functional SNPs and SNPs that previously associated in assays. Genotyping was performed by Illumina's GoldenGate genotyping system (http://www.illumina.com/General/pdf/LinkageIV/GOLDENGATE_ASSAY_FINAL.pdf).

Bioinformatics tools

SNPper (http://snpper.chip.org) using the National Center for Biotechnology Information (NCBI) Build 35.1 was used to determine marker positions (base pair (bp)), marker function, and identify amino acid changes. Kyoto encyclopedia of genes and genomes (KEGG) (http://www.genome.ad.jp/kegg/pathway.html) was used to examine gene ontology and to group genes into biological process pathways.

Statistical methods

Clinical and demographic characteristics between cases and controls were compared using Shapiro-Wilks tests of normality for gravidity (number of births), gestational age (days), gestational weight (grams (g)), APGAR 1, and APGAR 5. All measurements deviated significantly from normality; as a result Mann-Whitney two-sample rank sum tests were used to compare case and control groups (Hollander and Wolfe, 1999). Standard t-tests were used to test whether maternal age differed between cases and controls. χ^2 tests were used to test for differences in the counts of smokers and

non-smokers between cases and controls. STATA 9.0 statistical software (StataCorp, 2007) (College Station, TX, U.S.A) was used for all analyses.

Statistical tests for differences in single locus allele and genotype frequencies, deviations from Hardy Weinberg equilibrium (HWE), and measurements of inbreeding coefficients (F) were calculated using Powermarker statistical software (Liu and Muse, 2005) . Statistical significance for these analyses was determined using Fishers Exact tests. Measurements of population attributanle risk (proportion of cases in the study population that is attributable to the exposure) were calculated using Stata statistical software (StataCorp, 2007) (College Station, TX, U.S.A).

Pairwise LD was characterized and haplotype frequencies were calculated using Powermarker (Liu and Muse, 2005) and HaploView (Barrett et al., 2005) statistical software. Standard summary statistics D' and r^2 were calculated using HaploView (Zaykin et al., 2002). Haplotype blocks were assigned using the D' confidence interval algorithm created by Gabriel *et al.* (2002) (Gabriel et al., 2002). Both Powermarker and HaploView use an EM algorithm to determine haplotype frequency distributions when phase is unknown. The Powermarker haplotype trend analysis was performed for dichotomous outcome with 2, 3, and 4 marker sliding windows, using 10,000 permutations in order to determine p - values. This analysis is a regression approach to test haplotype-trait association. The test for association then uses an F test for a specialized additive model. The strongest associated sliding window was then analyzed for haplotype specific effects. This included the calculation of ORs for each haplotype, as well as determination of case and control haplotype frequencies. The highest frequency haplotype was used as the baseline haplotype frequency. Only haplotypes with

a frequency of 5% or more were considered for haplotype analyses and only significant haplotypes are reported.

Genes were grouped into KEGG biological process pathways and Z tests were used to determine if the total number of significant single locus allele and genotype associations within genes of a pathway statistically deviated from expected number of significant results given the number of tests and the dataset sample size. These analyses used only tag SNPs ($r^2 \geq 0.6$) to correct for lack of independence between markers within a gene due to LD.

Results are only presented for the following: 1) single locus tests of association with p values $\leq 10^{-3}$ for either allele or genotype tests of association; 2) haplotype tests for genes with at least 1 marker with a p values $\leq 10^{-3}$, genes with 3 or more markers; 3) Z-tests within KEGG pathways testing for deviations from expected number of significant tests (either allele or genotype) given the number of tests performed.

Exploratory multi-locus analyses were performed. Analyses were performed using Multifactor Dimensionality Reduction (MDR) which has been previously described in Ritchie *et al.* 2001 and is available as open source software at www.epistasis.org (Ritchie et al., 2001). First, all SNPs within the dataset, both for maternal and fetal data, were analyzed for two and three way interactions. The second analysis was conducted only on markers with statistically significant ($p \leq 0.05$) single locus effects within single KEGG pathways. In the context of this analysis MDR is being used as a search tool to identify interactions among loci with single locus associations. Markers were systematically removed if they were observed in one through three loci analyses and had a poor cross validation consistency (CVC) in all models; this was performed as a filtering

step to remove models due to strong single locus effects. All MDR analyses with statistically significant permutation tests were followed up with logistic regression analyses modeling the interaction. This was finally followed up with a likelihood ratio (LR) test modeling the interaction in logistic regression. Models were only considered significant if they were confirmed with logistic regression analyses. 10 fold cross validation was implemented with average balanced accuracy as the function for evaluating a model (Velez et al., 2007b).

Results

Baseline characteristics

Clinical and demographic characteristics are summarized in Chapter III part B on Table 3-8. Significant differences between cases and controls were observed for gestation age (days) ($p<0.001$), birth weight (grams) ($p<0.001$), APGAR 1 (1 minute after birth) ($p<0.001$), APGAR 5 (5 minutes after birth) ($p<0.001$), gravidity (number of births) ($p = 0.02$), and smoking ($p <0.001$).

Single locus associations

Table 4-1 presents the results for the single locus allele and genotype associations and ORs for the best model at each marker. The single strongest association in maternal data was seen at tPA rs879293 (allele $p = 2.30 \times 10^{-3}$; genotype $p = 2.00 \times 10^{-6}$) with a case minor allele frequency (MAF) = 0.35 and a control MAF = 0.46. The strongest model for the effects of this marker was GG v AG&AA (Table 4-1) with an OR = 2.80 [CI 1.77-4.44] and a $p = < 1.00 \times 10^{-6}$. Upon examining maternal genotypic associations, tPA at rs879293 remains significant after a Bonferroni correction.

In fetal samples the most significant association was (Table 4-1) seen in interleukin 10 receptor alpha (IL10RA) rs17121510 (allele p = 0.01; genotype p = $3.34x10^{-4}$) with case MAF = 0.15 and control MAF = 0.09. The most significant model for rs17121510 was AA v AG&GG with a protective OR = 0.43 [CI 0.25-0.74] (p = $2.00x10^{-3}$). In the fetal data the most significant ORs were for IL10RA rs17121510, klotho isoform a (KL) rs522796 (OR = 1.52 [CI 1.17-1.96]), and triggring receptor expressed on myeloid cells 1 (TREM1) rs6910730 (OR = 2.30 [CI 1.34-3.95]) all had equally significant results with p = $2.00x10^{-3}$; both KL rs522796 and TREM1 rs6910730 were additive models.

Model p-values for associated markers were adjusted for demographic variables that had baseline differences between cases and controls (Appendix Table 3). Both smoking and gravidity were used; however, gestation age, birth weight, APGAR 1, and APGAR 5 were not used as they are expected to differ between cases and controls. In maternal data all markers remained statistically significant after an adjustment, with the exception of coagulation factor V (FV) rs9332624, interleukin 5 (IL-5) rs739718, and prostaglandin E receptor 3 subtype EP3 isoform (PTGER3) rs977214. In fetal data all significance was lost with the exception of KL marker rs522796.

Given that the presence of MIAC in cases may influence associations we examined markers for allele and genotype difference between cases with and without MIAC (Appendix Table 4). There were no differences for any of the markers we examined in maternal or fetal data at either the allele or genotype level except fetal marker TREM1 rs6910730 that differed between MIAC and no MIAC at both the allele (p = 0.02) and genotype level (p = 0.05).

Among the markers with $p \leq 10^{-3}$ (this cutoff was chosen for presentation purposes and in order to focus on the strongest associations) seven deviated from HWE for either cases or controls (Table 4-1). Five were from maternal data, one in cases at tPA rs879293 (p = 0.01) and PTGER3 rs977214 (p = 0.02) and one in controls at IL-5 rs739718 (p = 0.02), tPA rs879293 (p = 0.01), and PTGER3 (p = 0.05). One was from fetal case data at IL-10RA marker rs17121510 (p = 0.02) and one was in fetal control data at cytathionine beta synthase (CBS) marker rs12329764 (p = 0.01). It is of note that for most situations where control $p < 0.05$ for HWE tests there is evidence that the inbreeding coefficients were in opposite directions in cases and controls. This suggests that the deviations are not likely due to genotyping error, as cases and controls were mixed in the same plates for genotyping.

Details regarding the associated markers are on Table 4-2a for maternal and Table 4-2b for fetal data. Among these markers, only one was a coding exon in fetal data (KL rs9527025, nonsynonymous amino acid change 370 S/C). The remaining markers were in promoters, introns, exons (synonymous), and exon/intron boundary regions.

Haplotype associations

Among the genes identified in single locus analyses, haplotypes in those genes were examined in five genes from maternal data (corticotrophin releaseing hormone binding protein (CRHBP), FV, IL-5, tPA, and PTGER3) and three from fetal data (CBS, IL-10RA, and TREM1). Detailed LD structure for maternal and fetal genes for cases and controls are available in Appendix Figure 3 a-p. Table 4-3 has the results of the haplotype association analyses with only the significant haplotypes and their ORs reported.

Table 4-1. Single locus association results and genotypic ORs

Population	*Gene(s)*	SNP rs#	Allele	Case Freq.	Control Freq.	Case v Control P		Model	OR	95% CI	Model P
						Allele	Genotype				
Maternal	*CRHBP*	rs1875999	G	0.29	0.40	$3.00x10^{-3}$	0.02	Additive	1.37	1.08-1.72	$8.00x10^{-3}$
		rs32897	G	0.13	0.21	$4.00x10^{-3}$	0.02	Additive	1.68	1.14-2.49	$9.00x10^{-3}$
		rs10055255	T	0.33	0.45	$5.90x10^{-4}$	0.01	AAvAT&TT	1.95	1.25-3.05	$3.00x10^{-3}$
	FV	rs9332624	C	0.03	0.01	$3.00x10^{-3}$	0.01	Additive	9.79	1.19-80.48	0.03
	IL-5	rs739718	G	0.09	0.04	$4.10x10^{-3}$	0.01	AAvAG&GG	0.38	0.19-0.79	$9.00x10^{-3}$
	tPA	rs879293*	A	0.35	0.46	$2.30x10^{-3}$	$2.00x10^{-6}$	GGvAG&AA	2.80	1.77-4.44	$<1.00x10^{-6}$
	PTGER3	rs977214	G	0.09	0.13	0.17	$4.08x10^{-3}$	AGvAA	0.51	0.29-0.90	0.02
		rs594454	G	0.44	0.31	$1.00x10^{-3}$	$4.00x10^{-3}$	Additive	1.71	1.24-2.35	$1.00x10^{-3}$
	SCNN1A/ sTNF-R1	rs3764874	G	0.28	0.19	$3.00x10^{-3}$	0.01	Additive	1.70	1.19-2.44	$4.00x10^{-3}$
Fetal	*CBS*	rs12329764	A	0.12	0.09	0.16	$2.00x10^{-3}$	GGvAG&AA	0.86	0.75-0.99	0.04
	IL-10RA	rs17121510	G	0.15	0.09	0.01	$3.34x10^{-4}$	AAvAG&GG	0.43	0.25-0.74	$2.00x10^{-3}$
	KL	rs9527025	C	0.10	0.18	$4.0x10^{-3}$	0.02	Additive	1.91	1.15-3.19	0.01
		rs522796	C	0.50	0.38	$3.00x10^{-3}$	$2.00x10^{-3}$	Additive	1.52	1.17-1.96	$2.00x10^{-3}$
	TREM1	rs6910730	G	0.15	0.07	$3.00x10^{-3}$	$3.00x10^{-3}$	Additive	2.30	1.34-3.95	$2.00x10^{-3}$

[1]maternal cases deviated from HWE at rs879293 (p = 0.01) andrs9772114 (p = 0.02) and fetal cases deviated at rs17121510 (p = 0.02)
[2]maternal control deviated from HWE at rs739718 (p = 0.02), rs879293 (p = 0.01), and rs977214 (p = 0.05) and fetal control deviated at rs12329764 (p = 0.01)
*Still significant after Bonferroni correction and FDR correction (false discovery rate = 0.20)

Table 4-2. Genes and markers with strongest associations

a. Maternal

Gene Name	*Gene Code*	Band[1]	# Markers Genotyped in Gene	dbSNP rs#	Role	Amino Acid Change	KEGG Pathway
Corticotrophin releasing hormone binding protein	*CRHBP*	5q23.3	5	rs1875999	Exon	-	-
				rs32897	Intron	-	
				rs10055255	Intron	-	
Coagulation factor V	*FV*	1q24.2	26	rs9332624	Intron	-	Compliment and coagulation cascade
Interleukin 5	*IL-5*	5q23.3	5	rs739718	3'UTR	-	T cell receptor signaling pathway/, Fc epsilon RI signaling pathway, hematopoietic cell lineage, Jak-STAT signaling pathway, cytokine-cytokine receptor interaction
Tissue plasminogen activator	*tPA*	8p11.21	8	rs879293	Intron	-	Compliment and coagulation cascade
Prostaglandin E receptor 3, subtype EP3 isoform	*PTGER3*	1p31.1	53	rs977214	Intron	-	Calcium signaling pathway, neuroactive ligand-receptor interaction
				rs594454	Intron	-	
Sodium channel, nonvoltage-gated 1 alpha/ Soluble tumor necrosis factor receptor 1	*SCNN1A/ sTNF-R1*	12p13.31	1	rs3764874	Intron (boundary) /Promoter	-	Taste transduction, cytokine-cytokine receptor interaction

b. Fetal

Gene Name	***Gene Code***	**Band**[1]	**# Markers Genotyped in Gene**	**dbSNP rs#**	**Role**	**Amino Acid Change**	**KEGG Pathway**
Cystathionine-beta-synthase	CBS	21q22.3	16	rs12329764	Intron (boundary)	-	Glycine, serine and threonine metabolism, methionine metabolism, Huntington's disease, selenoamino acid metabolism
Interleukin 10 receptor, alpha precursor	IL-10RA	11q23.3	11	rs17121510	3'UTR	-	Cytokine-cytokine receptor interaction, Jak-STAT signaling pathway
Klotho isoform b	KL	13.q13.1	19	rs9527025	Coding exon	370 S/C	-
				rs522796	Intron	-	
Triggering receptor expressed on myeloid cells 1	TREM1	6p21.1	11	rs6910730	Intron	-	-

[1]NCBI build 35.1
[2]Gene Ontology and KEGG pathway information obtained from SNPper (http://snpper.chip.org) and KEGG gene ontology browser

Table 4-3. Haplotype frequencies and OR for strongest sliding window

Population	*Gene(s)*	Markers	Global P[1]	Haplotype	Frequency		OR	95% CI	P
					Case	Control			
Maternal	*CRHBP*	rs32897-rs6453267-rs10055255-rs1875999	0.01	A-G-A-A	0.65	0.53	1.00	-	-
				A-G-T-G	0.10	0.18	0.45	0.27-0.74	9.00×10^{-4}
	FV	rs12131397-rs9332624-rs9332618	$<1.00 \times 10^{-3}$	C-A-C	0.43	0.52	1.00	-	-
				A-A-C	0.44	0.34	1.57	1.11-2.21	8.00×10^{-3}
	IL-5	rs739719-rs739718	0.01	G-A	0.90	0.94	1.00	-	-
				T-G	0.09	0.03	2.70	1.30-5.85	4.00×10^{-3}
	tPA	rs4471024-rs2020922-rs879293-rs2299609	6.00×10^{-3}	T-T-A-C	0.32	0.42	1	-	-
				C-A-G-G	0.29	0.21	1.82	1.20-2.76	3.00×10^{-3}
				T-T-G-C	0.21	0.17	1.62	1.03-2.55	0.03
	PTGER3	rs977214-rs6665776-rs594454	3.00×10^{-3}	A-C-T	0.564	0.469	1.00	-	-
				A-C-G	0.309	0.438	0.59	0.42-0.83	2.00×10^{-3}
	PTGER3	rs2050066-rs6424414-rs2300167	3.00×10^{-3}	C-C-C	0.314	0.402	1.00	-	-
				C-T-T	0.297	0.258	1.47	0.98-2.21	0.05
				C-C-T	0.286	0.209	1.76	1.15-2.67	6.00×10^{-4}
Fetal	*CBS*	rs1005584-rs6586282	9.00×10^{-3}	-	-	-	-	-	-
	CBS	rs1005584-rs6586282-rs6586283	9.00×10^{-3}	-	-	-	-	-	-
	IL-10RA	rs4938467-rs11216666	0.02	T-T	0.43	0.51	1.00	-	-
				C-C	0.16	0.08	2.31	1.33-4.04	1.00×10^{-3}
	TREM1	rs1817537-rs3804277-rs4711668	2.00×10^{-3}	G-G-C	0.48	0.48	1.00	-	-
				C-A-C	0.25	0.16	1.61	1.04-2.51	0.02

[1]Global P is the p-value for the haplotype sliding window

*Only haplotypes with frequencies of 5% in at least one status group and with a significant OR are presented.

*ORs are calculated comparing each haplotype to the highest frequency haplotype.

Among the haplotype sliding window analyses (sliding windows were performed with windows consisting of 2, 3, and 4 markers and only the strongest (most significant p value) window is presented) maternal FV had the most significant global p value ($<1.00x10^{-3}$) (Table 4-3; Appendix Figure 3g and h) associating intronic markers rs12131397-rs9332624-rs9332618 with PTB. The A-A-C haplotype (rs12131397-rs9332624-rs9332618; OR = 1.57 [CI 1.11-2.21], p $8.00x10^{-3}$) was the only significant haplotype. Two of these three markers (rs121313197 and rs9332618) are in tight LD (D` = 1) in both cases and controls (Appendix Figure 3c and 1d). The association does not appear to be main effect driven as rs9332624 is the only marker with a main effect but its MAF (0.03 in cases and 0.01 in controls) is low and does not appear to be contributing greatly to the haplotype association. tPA ($p = 6.00x10^{-3}$) and PTGER3 ($p = 3.00x10^{-3}$) both had strong global p values for haplotype sliding windows. The haplotype associations observed in tPA at rs4471024-rs2020922-rs879293-rs2299609 appear to be main effect driven by rs879293 as the rs879293 G allele was common to both associated haplotypes (C-A-G-G, OR = 1.82 [CI 1.20-2.76], $p = 3.00x10^{-3}$; T-T-G-C, OR = 1.62 [CI 1.03-2.55], p = 0.03) and the reference haplotype included A. Two non-overlapping haplotypes were associated in PTGER3, rs977214-rs6665776-rs594454 and rs2050066-6424414-rs2300167 both located in intronic regions. The pattern of haplotype associations suggests that both associations are main effect driven; the first haplotype is driven by the effect of rs594454 and the second haplotype by rs2300167.

Examining the three genes in fetal data (Table 4-3; Appendix Figure 3k-p), the associated haplotypes were in CBS (rs1005584-rs6586282 global $p = 9.00x10^{-3}$), CBS (rs1005584-rs6586282-rsrs6586283 global $p = 9.00x10^{-3}$), IL-10RA (rs4938467-

rs11216666 global p = 0.02), and TREM1 (rs1817537-rs3804277-rs4711668 global p = $2.00x10^{-3}$). In CBS only the global haplotype association was significant. IL-10RA had an associated haplotype C-C (rs4938467-rs11216666, OR = 2.31 [CI 1.33-4.04], p = $1.00x10^{-3}$) and this haplotype did not appear to be main effect driven. The TREM1 C-A-C (rs1817537-rs3804277-rs4711668, OR = 1.61 [CI 1.04-2.51], p = 0.02) haplotype associated with outcome. The TREM1, C-A-C (rs1817537-rs3804277-rs4711668), haplotype association appears to be driven by the effects of two marker C-A (rs1817537-rs3804277), as the rs4711668 C allele was common to the associated haplotype and the reference, suggesting that this marker does not contribute to the haplotype effect.

Table 4-4. Significant results by KEGG pathway

KEGG Pathway (tags with r2 ≥ 0.60)	# Genes	Population	
		Maternal	Fetal
Apoptosis	12		
Arachidonic acid metabolism	5		
Compliment and coagulation cascade	6	AG	
Cytokine-cytokine receptor interactions	31	A	AG
Focal adhesion	6	A	
Hematopoietic cell lineage	12	A	
Jak-STAT signaling pathway	14		
MAPK signaling pathway	18		
Neuroactive ligand-receptor interaction	12		
T cell receptor signaling pathway	12		
Toll-like receptor signaling	14		
Type I diabetes mellitus	7		
Other (genes not in other pathways)	55		AG

*A-indicates statistically significant allelic association
*G-indicates a statistically significant genotypic association

Pathway analysis

In maternal data the complement and coagulation pathway, cytokine-cytokine receptor interaction, focal adhesion, and hematopoietic cell lineage pathways had

significant excess of associations for either allele or genotype (Table 4-4). The complement and coagulation pathway had a significant excess of association for both allele and genotype tests. The cytokine-cytokine receptor and the other pathways were also significant for both allele and genotype tests for fetal data. None of the remaining pathways deviated from expected.

Multilocus analyses

Full data maternal multilocus analyses identified the tPA marker rs879293 as the single strongest model with 10/10 CVC with 59.94% average balanced accuracy (results not shown). Fetal full data multilocus analyses identified PTGFR marker rs6424776 and MTHFR marker rs1801133 as the best model with 9/10 CVC with 63.72 % (permutation $p = 0.02$); however, this model did not hold up to logistic regression analyses.

Multilocus analyses on pathways with excess of significant associations for either allele or genotype tests (identified by Z test analyses) revealed a statistically significant two locus model in the complement and coagulation pathway in maternal data after removing the strong main effect of tPA marker rs879293 (Figure 4-1a, b). This model included a marker in FV (rs2187952) and in coagulation factor VII (FVII) (rs3211719) and was confirmed with logistic regression analyses. The testing average balanced accuracy was 61.58% and the CVC was 10/10 (permutation $p < 1.00 \times 10^{-3}$). Logistic regression analyses showed that the interaction was statistically significant ($p = 9.00 \times 10^{-3}$) and the LR test p value for including the interaction in the logistic regression model was 7.20×10^{-3}.

a.

# of Loci	Model	Testing Balanced Accuracy (%)	Cross Validation Consistency (CVC)
1	rs2299609 (tPA)	53.40	6/10
2	rs2187952 (FV) x rs3211719 (FVII)	61.58	10/10
3	rs2187952 (FV) x rs3211719 (FVII) x rs4471024 (tPA)	50.03	3/10

b.

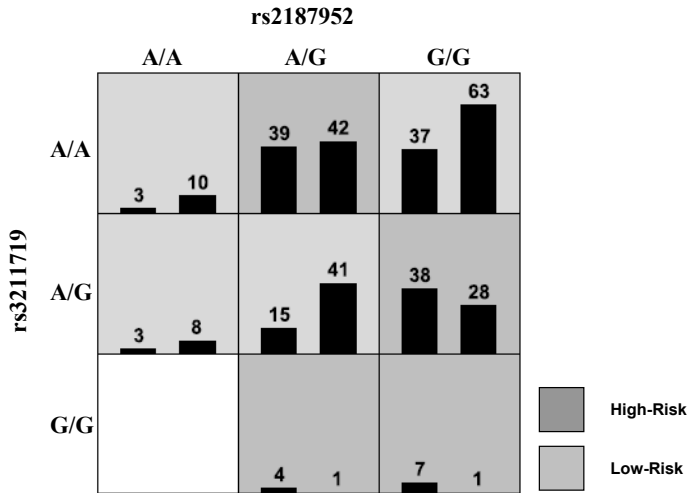


Figure 4-1. MDR compliment and coagulation two locus model between FV and FVII

Each multifactor cell is labeled as "high risk" or "low risk". For each multifactor combination, hypothetical distributions of cases *(left bar in cell)* and controls *(right bar in cell)* are shown. Each multilocus genotype is labeled on the figure. The testing average balanced accuracy is 61.58% (p-value $<1.00x10^{-3}$) with a CVC of 10/10. Logistic regression analyses showed that the interaction for the additive effects of these two markers was statistically significant ($p = 9.00x10^{-3}$) and the LR test p value for including the interaction in the logistic regression model was $7.20x10^{-3}$.

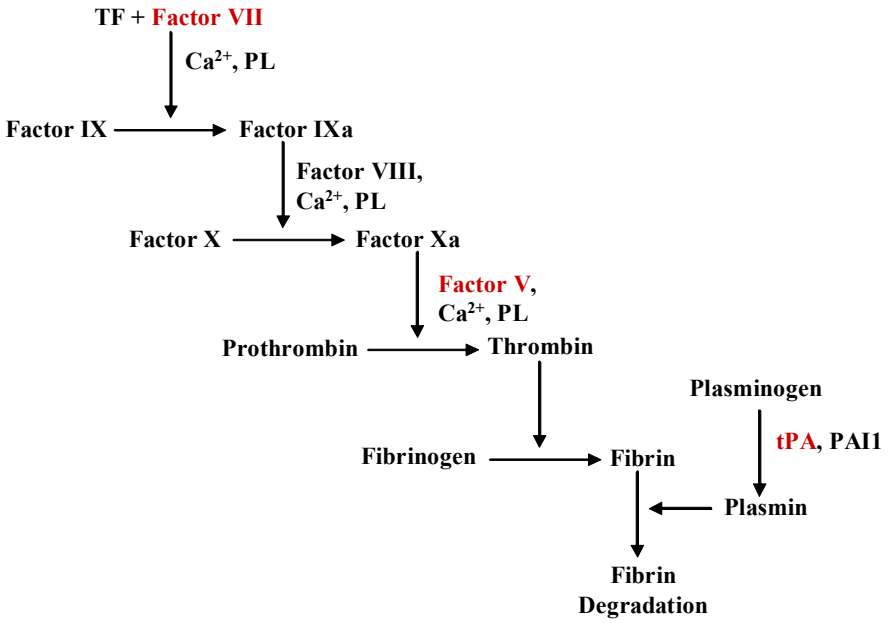


Figure 4-2. Compliment and coagulation pathway subset
This branch of the compliment and coagulation cascade had a cluster of three genes with significant ($\alpha = 0.05$) association at allele and/or genotype level, these are labeled in red (coagulation factor V (FV), coagulation factor VII (FVII), and tissue plasminogen activator (tPA)). In addition to these genes we also genotyped markers in three other genes from other branches of the compliment and coagulation cascade (soluble mannose-binding lectin 2 (MBL2), plasminogen activator inhibitor 1 (PAI1), coagulation factor II (FII) however, no markers in these genes were associated.

Discussion

In the present study a large-scale candidate gene association analysis was performed, in a U.S. Caucasian sample, on new and previously established PTB candidate genes in an effort to further understand the etiology underlying PTB. Comprehensive single locus, haplotype, and multi-locus analyses were performed and revealed several interesting results in both maternal and fetal data. Pathway analyses revealed strong associations in maternal data within the compliment and coagulation cascade, particularly in the fibrinolytic branch where three genes associated. Upon examining the compliment and coagulation pathway in more detail (Figure 4-2) it was

found that in the fibrinolytic branch of the pathway, three of four genes genotyped in that branch had markers that were significantly associated. These genes include coagulation factor VII (FVII), FV, and tPA. PAI1, also genotyped in that branch, was not significantly associated at the single locus allele or genotype level. Two other genes upstream and downstream of this branch did not associate (soluble mannose-binding lectin (protein C) 2 (MBL2) and coagulation factor II (FII)). The only result to stand up to a Bonferroni correction was tPA, from the compliment and coagulation cascade. The population attributable risk for this marker was ~0.32, suggesting that 32% of our cases can be explained by this variant. A multi-locus search also revealed an interesting association between FV and FVII within the compliment and coagulation cascade that suggests that there may be both an interaction among genes within the pathway and locus heterogeneity. Interestingly, the cytokine-cytokine receptor pathway revealed the strongest association with fetal DNA and it was also significant in maternal samples.

The majority of previous studies on PTB have focused on pathways contributing towards intrauterine infection (Lockwood, 2002) ignoring the potential contribution of other pathways such as decidual hemorrhage. Decidual hemorrhage is found in ~45% of patients with PTB (Salafia et al., 1995). Expression studies of decidual tissues have observed that tissue factors and tPA have strong patterns of expression (Bogic et al., 1999;Lockwood et al., 1993;Lockwood et al., 2001). To our knowledge there are no association studies associating tPA markers with PTB; however, several studies have observed that tPA and tissue factors are highly expressed in preterm human decidual tissues relative to term decidual tissues (Bogic et al., 1999;Bryant-Greenwood and Yamamoto, 1995). tPA is a serine protease inhibitor in the fibrynolitic cascade (Figure 4-

2) that converts inactive plasminogen to plasmin. The generation of plasmin is important for the degradation of components of the extra cellular matrix, such as matrix metalloproteinases (MMPs), which break down interstitial collagens. If MMPs are not degraded they can compromise the structural integrity of the decidua. Our association with tPA seen in maternal samples supports previous findings observing elevated amounts of tPA in maternally derived decidua and not in fetally derived amnion/chorion tissues (Bogic et al., 1999;Bryant-Greenwood and Yamamoto, 1995).

Another strong association was also observed in CRHBP in maternal data. Although it has not been directly associated with tPA it plays an important role in binding of corticotrophin releasing hormone (CRH). Studies have also demonstrated that tPA plays an important role in the cascade of CRH. CRH is released from several cell types, including but not limited to neuronal cells, the gastrointestinal tracts, placenta, and sites of inflammation; tPA is released in response to CRH and acts downstream of CRH receptor 1 (CRH-R1), a necessary step for the activation of anxiety-like behavior and neuronal activation (Matys et al., 2004). tPA is released from neurons upon excitation and is present in brain tissues such as the hippocampus, amygdala, hypothalamus, and cerebellum, functioning in neuronal plasticity (Baranes et al., 1998;Gualandris et al., 1996;Neuhoff et al., 1999;Parmer et al., 1997;Sappino et al., 1993). In the amygdala tPA has an important role in stress-induced synaptic plasticity and is an integral component for stress response (i.e. anxiety-like behavior) (Pawlak et al., 2002). This relationship is of interest as it would also implicate tPA with the maternal-stress PTB pathways in addition to decidual hemorrhage pathways. This may potentially implicate tPA and

CRHBP in maternal-stress pathways; however, despite the biological relationship between these two genes, no statistical associations were observed between them.

Single locus tests of association in fetal data were not as strong as maternal data and were not statistically significant after adjusting for the effects of confounders (gravidity and smoking) with the exception of KL rs522796. PTB with and without MIAC did not differ with the exception of TREM1 marker rs6910730. No statistically strong multilocus models were observed in any pathway in fetal data; however, a marginally significant model encompassing genes in PTGFR and MTHFR was observed with an average balanced accuracy of 63.72%. This result, however, did not hold up to logistic regression tests and one of the markers had a very rare genotype. The two largest pathways, the cytokine-cytokine receptor interaction pathway and the "other" pathway, were the pathways implicated with fetal data. The cytokine-cytokine receptor interaction pathway was also implicated in maternal data, which may be the result of either mother-fetal allele sharing or of independent associations in mother and fetus; however, such relationships can only be clarified by assessment of paternal contribution by the use of a family-based study design.

In conclusion, we conducted a gene-centric association study on PTB and found several interesting associations confirming several established PTB candidates, including a result that stood up to a Bonferroni correction. Sample size is a major limitation to our study, despite our sample being well phenotyped. However, given the patterns of associations observed within genes and by pathways, it is clear that several of the findings are consistent with established literature. We replicated the association found in FV, a well established PTB candidate also found using a large-scale high throughput

genotyping platform and identified new candidates (Hao et al., 2004). This association was previously observed in an African American population; however, our study suggests that it was not necessarily population specific given that we found the association in Caucasians. Our overall strongest association was seen in the decidual hemorrhage pathway suggesting that this pathway may be very important for the initiation of PTB and should be focused on in future studies.

B. A Large Scale High-throughput Preterm Birth Candidate Gene Association Study in African Americans

Introduction

The prevalence rate of PTB in the U.S. is ~17-18% for African Americans (Martin et al., 2005). Given the complex and poorly understood etiology underlying PTB and the high rate in African Americans, we chose to perform a large-scale PTB candidate gene association study focusing on an African American population. Data from previously published studies in both *in vivo* and *in vitro* human and animal models suggests that four primary pathogenic pathways either independently or through interactions lead to PTB (Lockwood and Kuczynski, 2001). All pathways culminate in a common terminal pathway that causes the release of uterotonins, such as prostaglandins, and eventually PTB (Lockwood and Kuczynski, 2001). We examined 1536 single nucleotide polymorphisms (SNPs) in and around 130 new and previously selected candidate genes from the hypothesized pathways described by Lockwood *et al.* (2001) in maternal and fetal samples. Single locus, haplotype, and multi-locus tests of association

were performed and genes were grouped according to known biological pathways (PTB – case; term birth – controls). Pathway grouping was based solely on biological function.

Materials and Methods

Study population

African American subjects from Centennial Women's Hospital in Nashville, TN were used in the present study. Please refer to Chapter III part A for a complete description of subject recruitment and ascertainment.

Demographic and clinical characteristics

Our study included African American maternal and fetal samples (75 case and 191 control maternal; 65 case and 183 control fetal). Placental pathology was performed in all cases to document histologic chorioamnionitis and funisitis. Smoking status (current smoker or not current smoker) was determined from a questionnaire.

Please refer to Chapter IV part A for a complete description of DNA sampling and genotyping, statistical analysis, and bioinformatics tools used in these analyses.

Results

Baseline characteristics

Clinical and demographic characteristics are summarized in Chapter III part B on Table 3-8. Significant differences between cases and controls were observed for gestation age (days) (0.001), birth weight (grams) ($p < 0.001$), APGAR 1 (1 minute after birth) ($p < 0.001$) and APGAR 5 (5 minutes after birth) ($p < 0.001$). A marginally significant difference was seen between case and control smokers ($p = 0.08$).

Single locus associations

Markers with the strongest associations ($p \leq 10^{-3}$) for either maternal or fetal samples are presented. Four of twenty-one markers with significant associations deviated from HWE (Table 4-5). Two were in maternal samples, one in cases at heat shock 70kDa protein 1-like (HSPA1L) rs2075800 ($p = 3.60x10^{-5}$) and one in controls at interleukin 2 receptor alpha (IL-2RA) rs6602392 ($p = 0.04$). Two were in fetal cases at CD14 antigen precursor (CD14) marker rs4914 ($p = 0.02$) and PON2 ($p = 0.01$). The deviations from HWE in cases may provide further support of an association at that marker. The inbreeding coefficients for cases and controls for the maternal IL-2RA marker rs6602392 had opposite signs, suggesting that the deviation from HWE was in opposite direction and not likely due to genotyping error.

Table 4-5 presents the results for the single locus allele and genotype associations and ORs for the best model at each marker. The single strongest association in maternal data was seen at interleukin 15 (IL-15) rs10833 (allele $p = 2.91x10^{-4}$; genotype $p = 2.0x10^{-3}$) with a case MAF = 0.06 and a control MAF = 0.18. The most significant maternal genotypic OR were seen for IL-15 rs10833 (OR = 0.54 [CI = 0.37-0.78] ($p = 1.0x10^{-3}$)) and interleukin 1 receptor accessory protein isoform (IL-1RAP) rs9290936 (OR = 1.99 [CI = 1.30-3.04] ($p = 1.0x10^{-3}$)), both are for additive models.

In fetal samples (Table 4-5) the single most significant association was seen in interleukin 2 receptor beta (IL-2RB) rs84460 (allele $p = 1.37x10^{-4}$; genotype $p = 6.29x10^{-4}$) with the case MAF = 0.25 and a control MAF = 0.44. The best model for rs84460 was additive with an OR = 2.36 [CI = 1.49-3.73] ($p<1.00x10^{-3}$). TNF-α marker rs1800629 (a.k.a. G-308A) has been previously associated with PTB, therefore, we examined it for

association as well and observed a marginally significant association (allele p = 0.01; genotype p = 0.02) in fetal data at this marker but not in maternal data, no deviations from HWE were seen (data not presented). Fetal genotypic ORs show the strongest ORs for IL-2RB rs84460 additive model (OR = 2.36 [CI 1.49-3.73] ($p = <1.0x10^{-3}$)), matrix metalloproteinase 2 (MMP2) rs243832 model GG v (CG&CC) (OR = 2.97 [CI = 1.60-5.54] ($p = 1.0x10^{-3}$)), and TNF-α rs1800683 additive (OR = 0.49 [CI = 0.32-0.76] ($p = 1.0x10^{-3}$)). Of note, this SNP is in strong LD with established PTB candidate -308 (rs1800629).

A description of the associated markers and gene functions is on Table 4-6a and Table 4-6b for maternal and fetal data. In maternal data HSPA1L rs2075800 (nonsynonymous amino acid change 602 E/K) resulted in a synonymous amino acid changes. The remaining markers were promoters, introns, exons (no amino acid changes), and exon/intron boundary regions.

Table 4-5. Single locus association results and genotypic ORs

Population	*Gene(s)*	SNP rs#	Allele	Case Freq.	Control Freq.	Case v Control P		Model	OR	95% CI	Model P
						Allele	Genotype				
Maternal	*HSPA1L*	rs2075800[1]	A	0.07	0.09	0.61	$5.93x10^{-4}$	AAvAG&GG	10.7	1.18-97.39	0.04
	IL-15	rs10833	A	0.06	0.18	$2.91x10^{-4}$	$2.00x10^{-3}$	additive	0.54	0.37-0.78	$1.00x10^{-3}$
	IL-1RAP	rs9290936	T	0.37	0.23	$1.53x10^{-3}$	0.01	additive	1.99	1.30-3.04	$1.00x10^{-3}$
	IL-2RA	rs6602392[2]	A	0.13	0.23	$4.46x10^{-3}$	0.03	additive	0.51	0.30-0.85	0.01
	IL-6R	rs4553185	C	0.48	0.61	$4.49x10^{-3}$	0.01	additive	1.75	1.19-2.59	0.01
	sTNF-R2	rs5746053	A	0.20	0.11	0.01	$3.00x10^{-3}$	additive	0.51	0.30-0.86	0.01
Fetal	*CD14*	rs4914[1]	G	0.10	0.03	$2.51x10^{-3}$	0.02	additive	2.69	1.29-5.64	0.01
	CTLA4	rs16840252	T	0.10	0.25	$2.06x10^{-4}$	$4.00x10^{-3}$	CCvCT&TT	0.33	0.17-0.65	$1.00x10^{-3}$
		rs231777	T	0.16	0.29	$2.52x10^{-3}$	0.01	additive	0.49	0.29-0.81	0.01
	IL-2	rs2069771	T	0.16	0.30	$1.75x10^{-3}$	0.01	additive	0.48	0.29-0.80	0.01
	IL-2RB	rs84460	C	0.25	0.44	$1.37x10^{-4}$	$6.29x10^{-4}$	additive	2.36	1.49-3.73	$<1.00x10^{-3}$
		rs228947	T	0.17	0.31	$8.80x10^{-4}$	0.01	CCvCT&TT	2.45	1.35-4.43	$3.0x10^{-3}$
		rs3218315	T	0.18	0.30	$3.81x10^{-3}$	0.02	TTvCT&CC	2.32	1.28-4.19	0.01
		rs228954	G	0.42	0.57	$2.47x10^{-4}$	0.01	additive	1.47	1.14-1.89	$3.00x10^{-3}$
		rs2281094	T	0.14	0.26	$3.46x10^{-3}$	0.02	CCvCT&TT	2.29	1.24-4.23	0.01
	MMP2	rs243832	G	0.40	0.55	$3.54x10^{-3}$	$2.71x10^{-3}$	GGvCG&CC	2.97	1.60-5.54	$1.00x10^{-3}$
	MMP9	rs3918260	C	0.27	0.14	$1.37x10^{-3}$	$3.30x10^{-3}$	additive	2.18	1.35-3.54	$2.00x10^{-3}$
	PON2	rs2237585[1]	T	0.45	0.58	0.01	$2.70x10^{-3}$	TTvCT&CC	0.28	0.12-0.62	$2.00x10^{-3}$
		rs2299267	G	0.22	0.12	$3.92x10^{-3}$	0.01	additive	2.16	1.28-3.65	$4.0x10^{-3}$
	PTGER3	rs4147115	A	0.39	0.53	$4.75x10^{-3}$	0.01	additive	0.54	0.35-0.83	$4.00x10^{-3}$
	TNF-α	rs1800683	A	0.35	0.51	$6.33x10^{-4}$	$4.06x10^{-3}$	additive	0.49	0.32-0.76	$1.00x10^{-3}$

[1]maternal cases deviated from HWE at rs2075800 ($p = 3.60x10^{-5}$) and fetal cases deviated at rs4914 (p = 0.02) and rs2237585 (p = 0.01)
[2]maternal controls deviated from HWE at rs6602392 (p = 0.04)

Table 4-6. Genes and markers with strongest associations

a. Maternal

Gene Name	*Gene Code*	Band[1]	dbSNP rs#	Role	Amino Acid Change	KEGG Pathway
Heat Shock 70kDa Protein 1-like	*HSPA1L*	6p21.33	rs2075800	Coding exon	602 E/K	MAPK signaling pathway, antigen processing and presentation
Interleukin 15	*IL-15*	4q31.21	rs10833	Exon	-	Cytokine-cytokine receptor interaction, Jak-STAT signaling
Interleukin 1 Receptor Accessory Protein	*IL-1RAP*	3q28	rs9290936	Intron	-	Cytokine-cytokine receptor interaction, apoptosis
Interleukin 2 Receptor, Alpha	*IL-2RA*	10p15.1	rs6602392	Intron	-	Cytokine-cytokine receptor interaction, Jak-STAT signaling, hematopoietic cell lineage
Interleukin 6 Receptor	*IL-6R*	1q21.3	rs4553185	Intron	-	Cytokine-cytokine receptor interaction, hematopoietic cell lineage, Jak-STAT signaling
Tumor Necrosis Factor Receptor Superfamily, Member 1B	*sTNF-R2*	1p36.22	rs5746053	Intron, Exon/ intron boundary	-	Apoptosis, cytokine-cytokine receptor interaction, MAPK signaling pathway, adipocytokine signaling pathway

b. Fetal

Gene Name	*Gene Code*	Band[1]	dbSNP rs#	Role	Amino Acid Change	KEGG Pathway
Monocyte Differentiation antigen CD14	*CD14*	5q31.3	rs4914	Coding exon	367 L/L	Regulation of actin cytoskeleton, pathogenic Escherichia coli infection - EPEC, Hematopoietic cell lineage, pathogenic Escherichia coli infection - EHEC, MAPK signaling pathway, toll-like receptor signaling pathway
Cytotoxic T-lymphocyte-Associated Protein 4	*CTLA4*	2q33.2	rs16840252	Promoter	-	T cell receptor signaling pathway, cell adhesion molecules (CAMs)
			rs231777	Intron	-	
Interleukin 2	*IL-2*	4q27	rs2069771	Intron	-	Jak-STAT signaling pathway, type I diabetes mellitus, T cell receptor signaling pathway, cytokine-cytokine receptor interaction
Interleukin 2 Receptor, beta	*IL-2RB*	22q13.1	rs84460	Intron	-	Cytokine-cytokine receptor interaction, Jak-STAT signaling pathway
			rs228947	Intron	-	
			rs3218315	Intron	-	
			rs228954	Intron	-	
			rs2281094	Intron	-	
Matrix Metallopeptidase 2	*MMP2*	16q12.2	rs243832	Intron (boundary)	-	GnRH signaling pathway, leukocyte transendothelial migration
Matrix Metallopeptidase 9	*MMP9*	20q13.12	rs3918260	Intron (boundary)	-	
Paraoxonase 2	*PON2*	7q21.3	rs2237585	Intron	-	Gamma-Hexachlorocyclohexane degradation, bisphenol A degradation
			rs2299267	Intron	-	
Prostaglandin E Receptor 3	*PTGER3*	1p31.1	rs4147115	Intron	-	Calcium signaling pathway, euroactive ligand-receptor interaction
Tumor necrosis Facto Alpha	*TNF-α*	6p21.33	rs1800683	Promoter	-	Hematopoietic cell lineage,Fc epsilon RI signaling pathway, type II diabetes mellitus, cytokine-cytokine receptor interaction, natural killer cell mediated cytotoxicity, toll-like receptor signaling pathway, T cell receptor signaling pathway, MAPK signaling pathway, Apoptosis, adipocytokine signaling pathway, TGF-beta signaling pathway, type I diabetes mellitus

[1]NCBI build 35.1
[2]Gene Ontology and KEGG pathway information obtained from SNPper (http://snpper.chip.org) and KEGG gene ontology browser

Haplotype associations

One gene for the maternal data met the criterion for haplotype analysis (IL-6R) and eight in fetal data (CD14, cytotoxic T-lymphocyte-associate protein 4 (CTLA4), IL-2, IL-2RB, matrix metalloproteinase 9 (MMP9), paraoxonase 2 isoform 1 (PON2), prostaglandin E receptor 3 subtype EP3 isoform (PTGER3), and TNF-α). Detailed LD structure for maternal and fetal genes for cases and controls are available in supplemental material (Appendix Figure 4a-r). Table 4-7 has the results of the significant haplotype associations and their ORs.

Maternal IL-6R haplotype sliding window analyses (sliding windows were performed with windows consisting of 2, 3, and 4 markers and only the strongest (most significant p value) window is presented) (Table 4-7; Appendix Figure 4a and b) associated intronic markers rs4075015-rs4601580-rs4845618 (p = 0.02) with cases. Haplotypes A-T-G (rs4075015-rs4601580-rs4845618; $p < 10^{-3}$) and A-T-T (rs4075015-rs4601580-rs4845618; OR = 0.17 [CI 0.02-0.71], p = 0.01) were both significantly associated haplotypes. This result does not appear to be main effect driven as despite rs4075015 A allele being common to all three, it did not associate at the p 0.05 single locus allele or genotype level.

Examining the nine genes in fetal data (Table 4-7; Appendix Figure 4c-r), the strongest haplotype associations were seen in: CD14 (rs4914-rs2569190), CTLA4 (rs16840252-rs11571317-rs5742909), IL-2 (rs2069771-rs2069776-rs2069778), IL-2RB (rs84460-rs228945-rs228947), MMP9 (rs6104420-rs3918260), and TNF-α (rs1800683-rs2229094). Among these associated haplotypes it is unclear whether MMP9

(rs6104420-3918260) and TNF-α (rs18006830-rs2229094) are driven by the main effects of single markers. MMP9 (rs6104420-3918260) G-C (OR = 2.27 [CI 1.31-3.91] ($p = 2.0x10^{-3}$)) had the strongest associated haplotype and in TNF-α A-C (rs1800683-rs2229094; OR = 0.37 [CI 0.20-0.68], $p\ 1.0x10^{-3}$) had the strongest associated haplotype. The haplotype associated in MMP9 included a promoter and an intron and within TNF-α, a promoter (rs1800683) and coding marker (rs2229094; nonsynonymous amino acid change 13 R/C) are included. Haplotypes in PON2, PTGER3 and TNF-α haplotype 2 all had significance levels no larger than p = 0.02.

Table 4-7. Haplotype frequencies and OR for strongest sliding window

Population	*Gene(s)*	Markers	Global P[1]	Haplotype	Frequency		OR	95% CI	P
					Case	Control			
Maternal	*IL-6R*	rs4075015-rs4601580-rs4845618	0.02	T-T-T	0.30	0.31	1.00	-	-
				A-T-G	0.07	$<10^{-3}$	-	-	$<10^{-3}$
				A-T-T	0.02	0.08	0.17	0.02-0.71	0.01
Fetal	*CD14*	rs4914-rs2569190	0.01	C-G	0.49	0.60	1.00	-	-
				G-G	0.11	0.03	3.91	1.58-9.71	$1.00x10^{-3}$
	CTLA4	rs16840252-rs11571317-rs5742909	$3.00x10^{-3}$	C-T-C	0.88	0.73	1.00	-	-
				T-T-C	0.10	0.22	0.37	0.18-0.71	$1.00x10^{-3}$
	IL-2	rs2069771-rs2069779-rs2069778	0.01	C-C-C	0.82	0.66	1.00	-	-
				T-C-C	0.15	0.30	0.39	0.22-0.68	$<10^{-3}$
	IL-2RB	rs84460-rs228945-rs228947	$4.00x10^{-4}$	T-A-C	0.56	0.42	1.00	-	-
				C-G-T	0.16	0.26	0.45	0.25-0.80	$4.00x10^{-3}$
				C-G-C	0.09	0.17	0.40	0.18-0.80	0.01
	MMP9	rs6104420-rs3918260	0.02	A-T	0.67	0.76	1.00	-	-
				G-C	0.24	0.12	2.27	1.31-3.91	$2.00x10^{-3}$
	PON2	rs2237585-rs11976060	$2.00x10^{-3}$	T-C	0.55	0.42	1.00	-	-
				C-C	0.45	0.58	0.61	0.40-0.93	0.02
	PTGER3	rs2050066-rs6424414-rs2300167-rs6678886	$2.00x10^{-3}$	T-C-T-A	0.40	0.39	1.00	-	-
				T-T-T-A	0.17	0.29	0.59	0.33-1.06	0.06
				T-T-T-G	0.11	0.05	2.12	0.92-4.75	0.05
	TNF-α	rs1800683-rs2229094	$2.00x10^{-3}$	G-T	0.59	0.45	1.00	-	-
				A-T	0.21	0.25	0.66	0.38-1.11	0.10
				A-C	0.13	0.27	0.37	0.20-0.68	$1.00x10^{-3}$
	TNF-α	rs1800683-rs2229094-rs1799964-rs1800629	$2.00x10^{-3}$	G-T-T-G	0.39	0.33	1.00	-	-
				A-C-C-G	0.11	0.19	0.51	0.25-1.00	0.04
				A-C-T-G	0.02	0.07	0.27	0.05-0.93	0.03

[1]Global P is the p-value for the haplotype sliding window

*Only haplotypes with frequencies of 5% in at least one status group and with a significant OR are presented.

*ORs are calculated comparing each haplotype to the highest frequency haplotype.

Table 4-8. Significant results by KEGG pathway

KEGG Pathway (tags with $r^2 \geq 0.60$)	# Genes	African American	
		Mother[A]	Fetal[A]
Apoptosis	12		
Arachidonic acid metabolism	5		
Compliment and coagulation cascade	6		
Cytokine-cytokine receptor interactions	31	AG	AG
Focal adhesion	6		
Hematopoietic cell lineage	12		A
Jak-STAT signaling pathway	14		AG
MAPK signaling pathway	18		
Neuroactive ligand-receptor interaction	12		
T cell receptor signaling pathway	12		AG
Toll-like receptor signaling	14		
Type I diabetes mellitus	7		
Other (Genes not in other pathways)	55		

*A indicates statistically significant allelic association
*G indicates a statistically significant genotypic association

Pathway analysis

All 130 genes were grouped into KEGG biological process pathways (Table 4-8); we included in our analyses all pathways with at least 5 genes and allowed for genes to be included in more than one pathway. Globally, across all single locus allelic analyses, both maternal and fetal samples had more significant allelic association tests than would be expected given the number of tests performed (Table 4-8). In fetal data, the cytokine pathway, T cell receptor signaling, and Jak-STAT signaling were significant for both allele and genotype tests; the hematopoietic cell lineage was significant for only allelic tests.

There was much overlap between genes across pathways. Examining the genes in the cytokine pathway, there were some genes unique to either maternal data or fetal data. For example, chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 8 (CCL8), interleukin 1 beta (IL-1β), interleukin 4 receptor (IL-4R), and interleukin 6

receptor (IL-6R) were associated in maternal but not in fetal data; while, interleukin 10 (IL-10), interleukin 1 receptor type II (IL-1R2), interleukin 2 (IL-2), interleukin 8 (IL-8), and tumor necrosis factor alpha (TNF-α) were associated in fetal but not maternal data. In the cytokine pathway maternal data had five genes with single locus associations' $\leq 10^{-3}$ (IL-1RAP, IL-2RA, IL-15, IL-6R and sTNF-R2). Fetal samples had three (IL-2, IL-2RB, TNF-α) genes with single locus associations $\leq 10^{-3}$ in the cytokine pathway, three in the hematopoietic cell lineage pathway (IL-1R2, TNF-α, and CD14), two in the Jak-STAT signaling pathway (IL-2 and IL-2RB), and three in the T cell receptor signaling pathway (IL-2, TNF-α, CTLA4).

All of the genes within fetal associated pathways, with the exception of the neuroactive ligand-receptor binding pathway, overlap with the cytokine pathway suggesting that the strongest associated pathway is in the cytokine pathway. Examining each of the non-cytokine pathways in detail did not reveal any clear within pathway patterns of association in maternal or fetal data.

a.

# of Loci	Model	Testing Averaged Balanced Accuracy (%)	Cross Validation Consistency (CVC)
1	rs1800890 (IL-10)	51.18	4/10
3	rs3024498 (IL-10) x rs1800683 (TNF-α)	62.46	8/10

b.

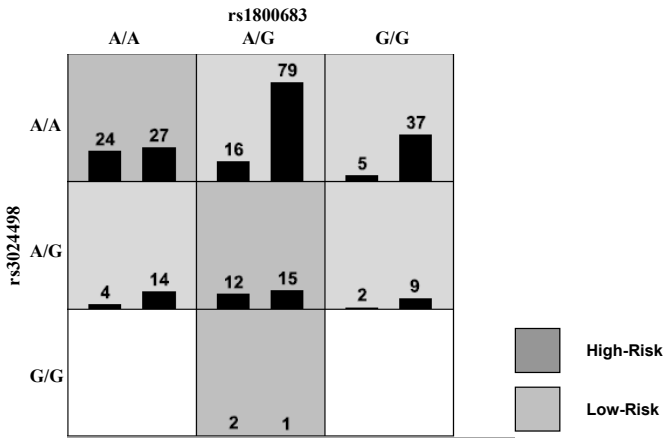


Figure 4-3. MDR T cell receptor two locus model between TNF-α and IL-10
Each multifactor cell is labeled as "high risk" or "low risk". For each multifactor combination, hypothetical distributions of cases *(left bar in cell)* and controls *(right bar in cell)* are shown. Each multilocus genotype is labeled on the figure. The testing average balanced accuracy is 62.46% (p-value = 0.03) with a cross-validation consistency of 8/10. Logistic regression analyses showed that the interaction for the additive effects of these two markers was statistically significant (p = 0.02) and the LR test p value for including the interaction in the logistic regression model was 0.02

Multi-locus analysis

Multilocus analyses examining the full dataset did not reveal any statistically significant multilocus associations; however, analyses within pathways with an excess of statistically significant associations (based on Z tests from pathway analyses) revealed a statistically significant two-locus model in the T cell receptor-signaling pathway in fetal data (Figure 4-3a, b). This model included a marker in TNF-α (rs1800683) and in IL-10 (rs3024498) and was confirmed with logistic regression analyses. The testing average

balanced accuracy was 62.46% and the CVC was 8/10 (permutation p = 0.03). The LR test showed that the interaction model (p = 0.02) was statistically significant.

Discussion

The present study examined multiple new and previously established PTB candidate genes in efforts to elucidate the mechanisms underlying PTB in African Americans. We observed multiple interesting single locus and haplotype associations in both maternal and fetal data and confirmed previously established candidates. In addition, pathway analyses demonstrated the strongest associated pathways related to infection and inflammation in both maternal and fetal data. A two-locus interaction was found in markers of the T cell receptor-signaling pathway of fetal data. These findings suggest that the documented elevated rates of infection seen in African American PTB may have a genetic basis (Goldenberg and Andrews, 1996;Hitti et al., 1997).

Single locus and haplotype associations revealed several interesting findings. Three coding variants associated in single locus and haplotype tests of association. Single locus associations identified a nonsynonymous amino acid change (602 E/K) in HSPA1L maternal samples, while haplotype associations identified a nonsynonymous amino acid change (13 R/C) in TNF-α fetal samples. Given that these coding variants result in nonsynonymous amino acid changes, they are likely to contribute to functional effects. One haplotype was associated in maternal data (IL-6R) and nine were associated in fetal data (CD14, CTLA4, IL-2, IL-2RB, MMP9, PON2, PTGER3, and two in TNF-α). Of note, one of the associated haplotypes in TNF-α (rs1800683-rs2229094-rs1799964-rs1800629) included a well established PTB candidate -308 (rs1800629) (Aidoo et al.,

2001). TNF-α -308 also had a marginally significant main effect in fetal samples but it did not associate in maternal data even at the marginal level.

Replication of TNF-α -308 associations in maternal data has been inconsistent across studies (Kalish et al., 2004). Despite this, elevated levels of TNF-α have been observed in PTB (Macones et al., 2004;Menon et al., 2006a), supporting a role for this gene in African Americans. It may be that fetal tissues rather than maternal tissues are contributing to the elevated TNF-α levels. A large cohort study by Aidoo *et al.* 2001 associated TNF-α -308 with fetal genotype in PTB, malaria, and with increased fetal mortality (Aidoo et al., 2001). Their results suggest that -308 may have a genetic effect but with fetal genotype rather than maternal. Our results are consistent with a fetal -308 single marker genotype effect as well as a haplotype specific effect.

The success of a pregnancy is highly dependent on the suppression of maternal immunity and attack by fetal alloantigens. Present understanding of PTB is limited and it is unclear as to what interactions may be occurring between maternal and fetal genetic information. In our study we observed several associations in genes and their receptors across maternal and fetal data that suggest interactions may be occurring between maternal and fetal genetic information. We observed that in maternal samples IL-2RA and sTNF-R2 associated with PTB and in fetal samples IL-2, IL-2RB, and TNF-α associated with PTB. These results are not necessarily surprising as organ explant studies suggest that both IL-2 and TNF-α are released by the amniochorion and not the maternal membranes (Aidoo et al., 2001) and other studies support that IL-2RA is regulated by the mother (Aidoo et al., 2001;Menon and Fortunato, 2004). With regard to TNF-α, it is regulated by complex interactions with its receptor during pregnancy. Elevated levels of

sTNF-R2 are usually the result of expression by cells of the immune system and are elevated in several inflammatory disorders (Zoller et al., 2007), while sTNF-R1 has typically been thought to play a role in pregnancy maintenance. The association with sTNF-R2 further supports the contention that infection may contribute to PTB in African Americans. Another gene PTGER3 (associated in fetal data) is expressed in the amnion but biological interaction between it and IL-1RA has been previously observed and suggests a maternal-fetal interaction may be occurring (Bry et al., 1993;Hirsch et al., 1999;Waleh et al., 2004). IL-1RA is associated in maternal data and is known to cause reduced prostaglandin production in the amnion/chorions (Bry et al., 1993;Hirsch et al., 1999;Waleh et al., 2004). Our results suggest interplay between these receptors and cytokines in the maternal-fetal unit may be in response to infection (Hirsch et al., 1999).

Several interesting results were observed in fetal data. The majority of the genes identified are known to be expressed in fetal tissue and are related to infection and/or inflammation during pregnancy. Studies examining CD14 (Hartel et al., 2004;Henneke et al., 2003;Kalish et al., 2006), CTLA4 (Schaub et al., 2006), IL-2 (Menon and Fortunato, 2004), MMP2 (McLaren et al., 2000;Tu et al., 1998), MMP9 (McLaren et al., 2000;Tu et al., 1998), PON2 (Chen et al., 2004a), PTGER3 (Waleh et al., 2004), and TNF-α (Menon and Fortunato, 2004) have observed the expression of these genes in fetal tissues (amnion/chorion) and little or no expression in the decidua. These data and our associations together support a genetic basis for the observed alterations in expression levels of these genes in fetal tissues in the presence of PTB.

Despite basing our candidates on hypothetical PTB pathways, we analyzed genes according to biological processes and identified the cytokine/infection/inflammation

pathway as the strongest associated pathway. This is a very promising finding given that African Americans are known to have a higher susceptibility to infection during pregnancy. In addition to this we observed the strongest number of associations with fetal genotype rather than with maternal genotype, suggesting that the fetal genetic contribution to PTB may exceed the maternal contribution. Sample size is a major limitation to our study; however, given the patterns of associations observed within genes and by pathway, it is clear that several of the findings are consistent with established literature.

In conclusion, we conducted an association study on PTB in an African American population and found several interesting association confirming several established PTB candidates. We observed several cytokines and receptors to associate across maternal and fetal data that suggest that a maternal-fetal exchange of genetic information may be contributing to the observed PTB. We also identified the cytokine infection and inflammatory response pathway as having the largest number of statistically significant associations across maternal and fetal data. Finally, we found a promising interaction between TNF-α and IL-10. These studies, although not conclusive did identify several interesting candidates for follow-up in replicate studies. Although many of the genes identified in Chapter IV part A overlap in the Caucasian population the majority of the genes identified here were those that participate in PTB through infection and inflammatory response pathways, in contrast to Caucasians were associations were also seen in pathways involved in decidual hemorrhage. These results suggests that many of the genetic factors that lead to PTB overlap across ethnic populations but mostly with regard the infection and inflammatory response pathways.

CHAPTER V

EXAMINING MULTILOCUS INTERACTIONS BETWEEN PRETERM BIRTH CANDIDATE GENES

Introduction

No studies have been performed comprehensively examining multilocus interactions in PTB on a large scale. In the present study we sought to address this deficiency by searching for epistatic interactions between PTB candidate genes in Caucasian and African American maternal and fetal populations and for maternal-fetal data. Multilocus interactions were analyzed by the use of Multifactor Dimensionality Reduction (MDR) (Hahn et al., 2003;Ritchie et al., 2001), a nonparametric approach developed to detect epistatic interactions in the absence of main effects that accepts case-control categorical and binary data (Hahn et al., 2003;Ritchie et al., 2001). Statistical significance of an MDR model was calculated by permutation testing.

Maternal-fetal interactions were examined by doubling the dimensions of the MDR model. For example, if 27 loci were genotyped 54 dimensions would be assessed (27 from mother and 27 from fetus). We also performed analyses within pathways, as previous studies suggest that this is a reasonable approach to reduce noise in an MDR analysis (Williams et al., 2004). We examined pathways individually and all pairwise combinations of pathways (defined by KEGG biological pathways). After performing all multilocus analyses, no single analysis produced a result that could be considered a strong multilocus model for PTB. However, promising results were seen in both

Caucasian maternal and African American fetal samples that could be followed up in future studies.

Materials and Methods

Study population, DNA sampling and genotyping

The study sample and experimental detail are provided in Chapter III part B. Demographic variables for the Caucasian and African American populations analyzed in the present study are described on Table 3-8.

Multifactor Dimensionality Reduction (MDR) and analyses performed

MDR. In the first step of MDR, the dataset is divided into multiple partitions for cross validation (CV) (Ritchie et al., 2001). Five-fold CV was used in the present analyses as studies suggest that this is a powerful and computationally efficient approach to find the best model (Motsinger and Ritchie, 2006). In five-fold CV, the training set is comprised of 4/5 of the data, while the testing set is comprised of the remaining 1/5 of the data. Second, a set of variables (i.e. SNPs) is selected. MDR exhaustively searches all single locus and all multilocus combinations of the data and collapses data into high and low risk categories on the basis of the ratio of cases to controls in each cell. It ultimately selects one genetic model, either single or multilocus, that most successfully predicts class or phenotype.

The final model is chosen from this set of models based upon maximization of testing accuracy and CVC. When prediction accuracy and CVC indicate different models, parsimony is used to choose the simplest model (the model with the fewest loci) (Hahn et al., 2003).

The data consisted of an unbalanced ratio of cases to controls, therefore, balanced accuracy was used in place of accuracy for the analyses (Velez et al., 2007b). Balanced accuracy is statistically equivalent to accuracy when datasets are completely balanced and is calculated from the average of sensitivity (the ability of a test to correctly identify those who have the disease) and specificity (the ability of a test to correctly identify those who do not have the disease) of the MDR model. Open-source MDR software is available at MDR (available at www.epistasis.org).

MDR analyses performed. Multi-locus analyses were performed examining all SNPs within the dataset both within Caucasian and African American maternal and fetal data as well as exploratory multilocus analyses of significant pathways. A comprehensive list of the analyses performed is presented on Table 5-1. Two way interactions were examined in African Americans and two and three way interactions were examined in Caucasians. Analyses performed include: full data, pathways, pairwise pathways, and analysis with and without single marker main effects (for either allele or genotype tests of association). These analyses were followed by examination of maternal-fetal interactions both within the full data and within pathways. Finally, for the final portion of the analysis (described in Chapter IV part A) we considered only markers that had statistically significant single locus effects. All MDR analyses with statistically significant permutation tests were followed up with logistic regression analyses modeling the interaction. This was finally followed up with a likihood ratio (LR) test modeling the interaction in logistic regression. Models were only considered significant if they were confirmed with logistic regression analyses.

Table. 5-1. List of MDR analyses performed

Analyses Performed
Full data in African Americans (both within maternal and within fetal samples)
Full data in Caucasians (both within maternal and within fetal samples)
Full data in African Americans with only tag SNPs (both within maternal and within fetal samples)
Full data in Caucasians with only tag SNPs (both within maternal and within fetal samples)
SNPs with significant single locus association in African Americans (both within maternal and within fetal samples)
SNPs with significant single locus association in Caucasians (both within maternal and within fetal samples)
SNPs with significant single locus association in African Americans with only tag SNPs (both within maternal and within fetal samples)
SNPs with significant single locus association in Caucasians with only tag SNPs (both within maternal and within fetal samples)
No SNPs with significant single locus association in African Americans (both within maternal and within fetal samples)
No SNPs with significant single locus association in Caucasians (both within maternal and within fetal samples)
No SNPs with significant single locus association in African Americans with only tag SNPs (both within maternal and within fetal samples)
No SNPs with significant single locus association in Caucasians with only tag SNPs (both within maternal and within fetal samples)
Pathways in African Americans (both within maternal and within fetal samples)
Pathways in Caucasians (both within maternal and within fetal samples)
Pairwise pathways (both within maternal and within fetal samples) in Caucasians considering all markers within a pathway
Pairwise pathways (both within maternal and within fetal samples) in Caucasians considering all markers with a significant association
Pairwise pathways (both within maternal and within fetal samples) in African Americans considering all markers within a pathway
Pairwise pathways (both within maternal and within fetal samples) in African Americans considering all markers with a significant association
Maternal-fetal interactions in African Americans full data and within pathway
Maternal-fetal interactions in Caucasians full data and within pathway
Maternal-fetal interactions in African Americans, markers with significant associations in full data and within pathways
Maternal-fetal interactions in Caucasians, markers with significant associations in full data and within pathway
Maternal-fetal interactions in African Americans, no markers with significant associations in full data and within pathways
Maternal-fetal interactions in Caucasians, no markers with significant associations full data and within pathway
Significant pathways, only significant markers, and doing a systematic removal of markers with strong single locus associations

Table 5-2. Full data MDR analysis

Population	# of Loci	Best Model For Each Interaction	Average Balanced Accuracy (%)	CVC
Caucasian Maternal	1	rs879293 (tPA)	59.94	5/5
	2	rs879293 (tPA) x rs6424776 (PTGFR)	58.73	2/5
	3	rs879293 (tPA) x rs6424776 (PTGFR)x rs380691 (DHFR)	51.24	1/5
Caucasian Fetal	1	rs522796 (KL)	51.31	2/5
	2	rs6424776 (PTGFR) x rs1801133 (MTHFR)	61.78	4/5
	3	rs6424776 (PTGFR) x rs1801133 (MTHFR) x rs3794808(SLC6A4)	53.05	1/5
African American Maternal	1	rs5744068 (TLR8)	46.51	1/5
	2	rs3783950 (MMP2)xrs243836 (TSHR)	40.4	2/5
African American Fetal	1	rs243836 (MMP2)	46.23	1/5
	2	rs3788050 (CBS) x rs1061628 (sTNF-R2)	57.3	3/5

Results

Full data analyses (Table 5-2) identified a one locus model in Caucasian maternal samples (tPA rs879293 5/5 = CVC, and p value ~ 0.01) and a two locus model in Caucasian fetal samples (PTGFR rs6424776 and MTHFR rs1801133, CVC = 4/5 p ~ 0.01). The LR test for interactions in the Caucasian fetal model had a $p < 10^{-3}$; however, upon examination of the multilocus model it appears to be driven by the presence of a rare genotype in rs6424776. The models identified in African Americans were not statistically significant. Of note, PTGFR was common to models in Caucasian maternal and fetal samples and matrix metalloproteinase 2 (MMP2) was common to African American maternal and fetal samples, common associations in maternal and fetal samples may be due to maternal fetal sharing.

Table 5-3. Pathway MDR analysis

Population/ Pathway	# of Loci	Best Model For Each Interaction	Average Balanced Accuracy (%)	CVC
Caucasian Maternal (Compliment & coagulation)	1	rs879293 (tPA)	58.90	5/5
	2	rs9332575 (FV) x rs879293 (tPA)	57.43	3/5
	3	rs879293 (tPA) x rs2070682 (SERPINE1) x rs12131397 (FV)	52.80	1/5
Caucasian Fetal (Compliment & coagulation)	1	rs3211719 (FVII)	46.31	2/5
	2	rs2070852 (FII) x rs12131397 (FV)	54.02	3/5
	3	rs2070852 (FII) x rs1894697 (FV) x rs12131397 (FV)	55.14	3/5
African American Maternal (Type I diabetes)	1	rs4848306 (IL-1B)	53.06	3/5
	2	rs9658742 (FAS) x rs10027390 (IL-2)	49.20	3/5
African American Fetal (Apoptosis)	1	rs740841 (TNFRSF1A)	58.89	3/5
	2	rs740841 (sTNF-R1) x rs1800683 (sTNF-R1)	60.58	4/5

Table 5-4. Pairwise pathway MDR analysis

Population/ Pathway	# of Loci	Best Model For Each Interaction	Average Balanced Accuracy (%)	CVC
Caucasian Maternal (Compliment & coagulation & Arachidonic acid metabolism)	1	rs879293 (tPA)	58.90	5/5
	2	rs879293 (tPA) x rs9332575 (FV)	60.60	3/5
	3	rs879293 (tPA) x rs3820185 (PLA2G4A) x rs2227631 (SERPINE1)	53.83	2/5
Caucasian Fetal (Compliment & coagulation & T cell receptor signaling)	1	rs7674640 (NFKB1)	43.13	2/5
	2	rs2420369 (FV) x rs2070852 (FII)	50.12	1/5
	3	rs879293 (tPA) x rs7674640 (NFKB1) x rs1800890 (IL-10)	59.95	3/5
African American Maternal (Neuroactive ligand receptor & T cell receptor signaling)	1	rs12885526 (TSHR)	52.28	2/5
	2	rs6713532 (POMC) x rs10027390 (IL-2)	58.85	3/5
African American Fetal (Cytokine & Other)	1	rs2069771 (IL-2)	48.83	2/5
	2	rs3789204 (TREM1) x rs1061628 (sTNF-R2)	61.53	4/5

Pathway analyses (Table 5-3) with Caucasians maternal samples again identified tPA rs879293 5/5 = CVC. No other significant models were identified by pathway analyses in any other population. Analyses between pairwise pathways (Table 5-4) also identified tPA rs879293 within Caucasian maternal samples. A three locus model was also identified in Caucasian fetal samples (tPA rs879293, PLA2G4A rs7674640, and SERPINE1 rs2227631 CVC = 3/5 and p = 0.03). No significant models were found in African American maternal or fetal samples.

Table 5-5. Best results from MDR analysis of significant single locus associations and nonsignificant single locus associations both overall and within pathways

Population/ Pathway	# of Loci	Best Model For Each Interaction	Average Balanced Accuracy (%)	CVC
Caucasian Fetal (No significant single locus associations or genes with genes with significant single locus associations) Overall data	1	rs3211719 (FVII)	45.83	1/5
	2	rs6424776 (PTGFR) x rs1801133 (MTHFR)	56.11	4/5
	3	rs646474 (SERPINH1) x rs6424776 (PTGFR) x rs3917273 (IL-1R1)	53.06	1/5
African American Maternal (Significant single locus associations including all markers in gene) Other Pathway	1	rs17007610 (IL-15)	60.64	3/5
	2	rs1700610 (IL-15) x rs10027390 (IL-2)	65.01	3/5

No significant models were found in analyses by removing or including main effects from the datasets used in the MDR analysis (Table 5-5). Further analyses were performed examining main effects and systematically removing markers, and are presented in Chapter IV part A for Caucasians and part B for African Americans.

Table 5-6. Best results from MDR analysis of maternal-fetal interactions

Population/ Pathway	# of Loci	Best Model For Each Interaction	Average Balanced Accuracy (%)	CVC
Caucasians	1	Maternal rs879293 (tPA)	59.21	5/5
	2	Fetal rs17561(IL-1A) x Maternal rs879293 (tPA)	62.15	3/5
	3	Fetal rs7901656 (FAS) x Maternal rs879293 (tPA) x Maternal rs12131397 (FV)	52.34	1/5
African American	1	Fetal rs84460 (IL-2RB)	46.68	1/5
	2	Fetal rs7698675 (IL-15) x Maternal rs10027390 (IL-2)	64.95	4/5

Finally, multilocus analyses were performed examining maternal-fetal interactions. No models were found in African American samples; however, in Caucasian samples an interaction was found between fetal IL-1A rs17561 and maternal tPA rs879293 (CVC = 3/5 and $p < 10^{-3}$). The LR tests for this model had a p value 6.0×10^{-3}.

Discussion

A comprehensive multilocus analysis was performed in Caucasian and African American maternal and fetal samples including analyses of maternal-fetal interaction, in efforts to more clearly understand the etiology underlying PTB. Several marginally significant results were found with a few that were significant in logistic regression analyses, including a model in Caucasians examining maternal-fetal interaction (tPA rs879293 and IL-1A rs17561). We were underpowered for several of the analyses, in particular for African American maternal and fetal analyses and Caucasian and African

American maternal-fetal analyses. This may be the reason for weak permutation test p values and low CVC.

A major issue encountered in these analyses was the influence of main effects on multilocus analyses; almost all models identified in full data analyses included a strong main effect. For example, the strongest association we identified in single locus analyses, tPA rs879293, in Caucasian maternal data was in every model of Caucasian maternal analyses. As a result of this we performed several analyses both with and without main effects. Unfortunately, no models were found with strong CVC in these analyses. It is possible that MDR may not be appropriate for these analyses given that MDR was designed to detect interactions in the absence of main effects. The main effects that we identified in the multilocus analyses were the same main effects identified in single locus analyses of alleles and genotypes (Chapter IV part A and B).

Of note, we also performed full data analyses both within Caucasian and African American maternal and fetal data by selecting tag SNPs (r^2 less than or equal to 0.90). Selecting tags reduced the number of SNPs by a negligible amount and produced the same models identified in the full data analysis without selecting tags. We concluded that LD does not have great influence over the MDR analyses, this is not surprising given that markers were chosen, based on being tags in the representative populations of the HapMap.

We did identify some interesting multilocus associations in Caucasian maternal and African American fetal samples that are described in detail in Chapter IV part A and B. However, an issue regarding these analyses is that all of the analyses performed (greater than 800) increase the likelihood of finding a false positive which reduces our

confidence in these results. We cannot conclude that these were real multilocus interactions, despite the strong statistical evidence. To determine if these associations are not spurious false positive findings, future analyses will be performed in a larger powered dataset, with MDR analyses focused on the promising associations found in the present study.

CHATPER VI

MITOCHONDRIAL DNA VARIANT A4917G, SMOKING AND SPONTANEOUS PRETERM BIRTH

Overview

The exact cause of PTB is unknown but oxidative stress may play an important role. Genetic studies have recently begun to elucidate the role of genetic variation in PTB but these studies have overlooked the mitochondrial genome/gene(s) as a plausible PTB candidate. In the present study we sought to document associations between nonsynonymous mitochondrial DNA (mtDNA) variants A4917G, G10398A and T4216C and PTB. We performed a case (PTB - less than 36 weeks gestation) - control (normal term - ≥ 37 weeks) analysis of these mtDNA markers and examined their potential interaction with smoking in PTB. A sample of 422 pregnant Caucasian women (220 preterm and 202 terms) was examined for association. Haplogroup T marker A4917G was identified as a possible candidate for association with PTB after adjusting for smoking (OR = 1.99 [95% CI 0.93-4.24]) as was T4216C (OR = 1.63 [95% CI 0.93-2.83]). No significant multi-locus interactions or interactions with other environmental variables were observed. Our data, although preliminary, support the hypothesis that mitochondrial genome polymorphisms may play a significant role in PTB through an interaction with smoking.

(Chapter VI adapted from Velez et. al. "Mitochondrial DNA variant A4917G, smoking and spontaneous preterm birth." *Mitochondrion* In press.)

Introduction

Previous studies have found evidence indicating that PTB has a substantial genetic component (Romero et al., 2006;Romero et al., 2007;Varner and Esplin, 2005). However, genetic studies have focused on genes in a limited number of specific pathophysiological pathways proposed to affect risk of PTB (Lockwood and Kuczynski, 2001) and/or on SNP(s) thought to have functional effects based on *in vitro* analysis.

In the previous genetic studies, the mitochondrial genome has been overlooked despite the fact that it is a very plausible candidate (Agarwal et al., 2006). The mtDNA is of particular interest because oxidative stress is likely to play an important role in the risk of PTB. Specifically, previous studies have shown that pregnancy results in increased oxidative stress (Myatt and Cui, 2004) and that during labor, in particular, higher levels of serum hydroperoxides exist compared to women not in labor (Fainaru et al., 2002). Given that both cell apoptosis and oxidative stress during pregnancy may contribute to adverse pregnancy outcomes, it is logical to examine mitochondrial DNA (mtDNA) variants for association with PTB.

Other lines of evidence also support the hypothesis that mtDNA variation may contribute to PTB. For example, in the early stages of pregnancy environmental toxins can contribute to PTB via oxidative stress, imbalanced production of ROS and reactive nitrogen species (RNS) (Mohorovic, 2004). During the initial stages of pregnancy the fetus is unable to defend itself against hypoxia (Jaffe et al., 1997;Jaffe, 1998;Jauniaux et al., 2000) and the oxidative stress that increases apoptosis (Jaffe, 1998). Hypoxia in turn leads to disrupted maternal vascular endothelium, accumulation of endothelin, thromboxane, superoxides, an increased sensitivity to angiotensin II, and decreased

formation of vasodilators (i.e. nitric oxide) (Granger et al., 2002;Roberts, 1998;Roberts, 2000). High levels of lipid peroxides have been associated with PTB, suggesting an exposure to oxidized nitrogen compounds (Tabacova et al., 1997;Tabacova et al., 1998).

In the present study we decided to focus on three well established mtDNA variants that have been seen to associate with several phenotypes: A4917G, G10398A, and T4216C. A4917G is a well established marker of the haplogroup T, which has been associated with several phenotypes (Ruiz-Pesini et al., 2004;Tanaka et al., 1998). Both A4917G and T4216C (Ruiz-Pesini et al., 2000;Ruiz-Pesini et al., 2004) result in non-conservative amino acid changes that increase reactive oxygen species (ROS) production and electron leakage, directly leading to mtDNA and nuclear DNA mutations and dysfunction (Penta et al., 2001;Robinson, 1998;Smeitink and van den, 1999). These markers have been associated with Leber's Hereditary Optic Neuropathy (LHON) and Parkinson's disease (Fauser et al., 2002;Hofmann et al., 1997;Howell et al., 1995;Kirchner et al., 2000;Ross et al., 2001;Ross et al., 2003). In addition, G10398A has been associated with multiple disease phenotypes potentially related to oxidative damage, including Parkinson's disease, Alzheimer's disease, Friedreich's ataxia, and amyotrophic lateral sclerosis (Giacchetti et al., 2004;Ivanova et al., 1998;Mancuso et al., 2004;Ross et al., 2001;Shoffner et al., 1993;van der Walt et al., 2003;van der Walt et al., 2004). Since both apoptosis and oxidative stress are putative risk factors for PTB, we hypothesize that these non-conservative mtDNA polymorphisms in the subunits of Complex I of the electron transport chain associate with PTB. Given previous observed associations between smoking and PTB we decided to examine smoking for interactions with mtDNA

variants. In this study, we examine the association of the above described mitochondrial markers with PTB in white maternal DNA samples from preterm and term deliveries.

Materials and Methods

Study population

Caucasian subjects were recruited at the Centennial Medical Center, Nashville, TN and Magee Women's Hospital, Pittsburgh, PA. Please refer to Chapter III part A for a description of subject recruitment.

Demographic and clinical characteristics

Our study included 220 case and 202 control maternal-fetal paired samples. As mtDNA is maternally inherited, fetal genotype should be identical to maternal genotype. All maternal-fetal pairs were genotyped as a means of genotyping quality control. Smoking status was obtained by questionnaire as described earlier in Chapter IV.

DNA sampling and genotyping

DNA was isolated from maternal and cord blood samples, using the Autopure automated system (Gentra Systems (Minneapolis, MN). Genotyping was done using 5'nuclease allelic discrimination TaqMan assay on an ABI 7900 (Applied Biosystems of Foster City, CA). Three mtDNA single nucleotide polymorphisms (SNPs) were examined: A4917G, G10398A, and T4216C. Genotype data were analyzed using ABI Sequence Detection System version 2.1 software and confirmed by visual inspection of the plots. Genotypes were classified as undetermined if PCR amplification failed with the specified sets of probes and primers.

Statistical methods

Continuous demographic data were analyzed using Mann-Whitney two sample rank sum tests, as data were not normally distributed and categorical demographic data were analyzed using a χ^2 test. Both univariate and multivariate analyses were used to examine the association between markers and PTB. For univariate analyses χ^2 tests were used to compare allele frequencies between cases and controls. Since the mtDNA markers are all homoplastic (one allele present for an individual) a single test was performed. The major allele was treated as the baseline allele for χ^2 tests and calculations of OR/effect sizes with 95% confidence intervals. The major allele was A for A4917G and G10398A and T for T4216C. Multivariate logistic regression analyses were performed to assess the relationship between PTB and genetic variation, adjusting for smoking, as smoking was significant at the 0.05 level. Other potential confounders were assessed in preliminary analyses, including body mass index (BMI), years of education and income, but did not affect results and are not presented. STATA 9.0 statistical software (StataCorp, College Station, TX) (StataCorp, 2007) was used for both univariate and multivariate analyses.

Results

The medians and interquartile ranges of demographic and clinical characteristics for cases and controls are listed in Table 6-1. Significant differences between cases and controls were observed for birth weight, APGAR 1, APGAR 5, and gestational age at delivery (weeks) (as expected) with p-value <0.01. Smoking was significantly associated with PTB ($p < 0.01$).

Table 6-1. Demographic characteristics

Variable	Caucasian				
	Control (N=202)		Case (N=220)		[1]P-value
	Median	[IQR]	Median	[IQR]	
Gestational Age	39	[37-42]	33	[23-36]	<0.01
Gestational Weight (g)	3433	[370-4661]	1879	[370-3790]	<0.01
Maternal Age (yrs)	28	[18-40]	27	[18-40]	0.26
APGAR 1	8	[4-9]	8	[1-9]	<0.01
APGAR 5	9	[7-10]	9	[1-9]	<0.01
Smoking Status (Count)	15%		29%		<0.01

[1]P-value-refers to p-value after comparing term to preterm; if continuous data was not normally distributed and a Mann-Whitney two-sample rank sum test was used. A χ^2 test was used for comparisons of smoking counts.
*IQR-interquartile range

Single marker analysis of mitochondrial variants did not reveal statistically significant associations (Table 6-2). Given the strong association found between cases and controls for smoking, we performed logistic regression analyses to examine the interaction between smoking and the mtDNA variants (Table 6-3a, b, and c). We performed this analysis under the hypothesis that smoking may be interacting with genotype, as smoking confers an increased risk for PTB by increasing oxidative stress and has been seen to interact with genotype in several disease phenotypes. Our analyses did not reveal any statistically significant interactions with smoking or any of the mtDNA polymorphisms. However, upon adjusting for the effect of genotype x smoking interaction the main effects of both A4917G and T4216C trended towards significance, with p-values of 0.07 and 0.08, with ORs of 1.91 [95% CI 0.89-4.11] and 1.65 [95% CI 0.91-2.81], respectively.

Because of the strong effect of smoking in our sample a secondary analysis on non-smokers was performed (Table 6-4). In cases, both A4917G (Table 6-4a; OR 1.99 [95% CI 0.93-4.24]; p-value = 0.07) and T4216C (Table 6-4c; OR 1.63 [95% CI 0.93-

2.83]); p-value = 0.08) became marginally significant in non-smokers. C10398A (Table 6-4b) was still non-significant after removing smokers.

Table 6-2. Univariate data analysis A4917G, G10398A, and T4216C

Marker	Allele	Status		OR	OR 95% CI	P-value
		Case (N = 220)	Control (N = 202)			
A4917G	G	24	18	1.21	0.616-2.45	0.56
	A (reference)	193	175			
G10398A	G	43	33	1.27	0.75-2.18	0.35
	A (reference)	165	161			
T4216C	Caucasians	49	39	1.17	0.71-1.93	0.52
	T (reference)	171	159			

Table 6-3. Multivariate analysis adjusting for confounders and examining smoking by genotype interaction

a.

Variable	OR	95% Confidence Interval	P-Value
Smoking	2.57	1.48-4.48	<0.01
A4917G	1.91	0.89-4.11	0.07
Smoking*A4917G	0.33	0.06-1.89	0.21

b.

Variable	OR	95% Confidence Interval	P-Value
Smoking	2.70	1.48-4.92	<0.01
G10398A	1.22	0.66-2.54	0.53
Smoking*G10398A	0.59	0.15-2.37	0.46

c.

Variable	OR	95% Confidence Interval	P-Value
Smoking	2.73	1.53-4.49	<0.01
T4216C	1.65	0.91-2.81	0.08
Smoking*T4216C	0.40	0.10-1.58	0.19

Table 6-4. Multivariate analysis adjusting for confounders only using non-smokers

a.

Variable	OR	95% Confidence Interval	P-Value
A4917G	1.99	0.93-4.24	0.07

b.

Variable	OR	95% Confidence Interval	P-Value
G10398A	1.19	0.65-2.20	0.57

c.

Variable	OR	95% Confidence Interval	P-Value
T4216C	1.63	0.93-2.83	0.08

Discussion

A retrospective case-control association study was performed to examine genetic variations in several mtDNA related genes of complex I of the electron transport chain in an effort to understand their contribution to PTB. In addition, we assessed the interaction of these variants with smoking because smoking is a known risk factor for PTB. As expected, we observed a strong association between maternal smoking and PTB. Approximately 15% of white controls and ~29% white cases smoked (p=0.002), supporting a role for environmental sources of oxidative stress that may contribute to PTB by interacting with variations in the mtDNA. The strong association of smoking and PTB we observed OR 2.3-2.6 exceeds the effect sizes previously published (OR ~ 1.5) (Goldenberg and Andrews, 1996;Meis, 2005).

Both univariate and multivariate analyses adjusting for confounders were performed. Examination of interactions between smoking and mtDNA polymorphisms did not reveal any statistically significant associations; however, the main effects of A4917G and T4216C became marginally significant upon adjusting for the effect of interactions with smoking. Upon stratifying by smoking, non-smokers revealed two

marginally significant associations at A4917G and T4216C, further supporting the hypothesis that these two markers may influence susceptibility to PTB. Therefore, it is still likely that these markers have an effect on PTB when adjusted for smoking, especially given the OR of ~2.0. Interactions with smoking need to be considered in any analysis of mtDNA because smoking confers such a large increase in risk for PTB from oxidative stress that genetic effects related to oxidative damage may not be detected in the presence of smoking. If this is the case, and genetic factors that affect oxidative stress are much weaker, but still significant, than the potential environmental factors, then statistical association with gene variants will be overwhelmed in the presence of a factor such as smoking. Therefore, it will be important to assess the role of these variants in non-smokers only.

Post-hoc power calculations, using PS statistical software (Goldenberg and Andrews, 1996), show that our sample had 80% power to detect an OR of 1.87-2.19 with risk allele frequencies of 0.10 and 0.20 ($\alpha = 0.05$); therefore, a larger sample size may be required to detect a statistically more significant effect. Despite this, marginally significant effects for mtDNA polymorphisms were found after stratifying by smoking status. Although we cannot draw any strong conclusions as to the role of these markers in PTB, our results suggest that in genetic studies of PTB, relating to oxidative stress, stratifying by smoking status will be required to detect effects of mtDNA, as smoking is a powerful confounder that greatly influences the ability to detect effects. One caveat that we have for our study is that we only studied spontaneous preterm birth; other related phenotypes such as preterm premature rupture of the membrane (pPROM) and preeclampsia may be more related to oxidative stress (Agarwal et al., 2006). However,

we would argue that to define genetic risk factors one needs to carefully limit phenotypic heterogeneity and we have attempted to do this in our study (Longini et al., 2007).

Smoking, oxidative stress, and mutations in the mitochondrial respiratory chain can lead to PTB by disruption of placental mitochondrial function. Two studies by Bouhours-Nouet et al. (2004) and Wiktor et al. (2004) support this contention and found that the placenta was susceptible to oxidative damage that can lead to adverse pregnancy outcomes (Bouhours-Nouet et al., 2005;Wiktor et al., 2004). The study by Bouhours-Nouet et al. (2004), found that maternal smoking, in particular, is associated with respiratory chain complex deficiencies in the placenta. Smoking increases ROS production and can have direct effects on both trophoblast formation and amino acid uptake in the placenta, affecting amino acid uptake, transfer into the placenta, and synthesis (Bouhours-Nouet et al., 2005;Wiktor et al., 2004). Placental ROS mediated damage can either inactivate electron transport chain complexes or inhibit mtDNA transcription. Given that placenta amino acid uptake is carefully regulated by mechanisms dependent on the cellular formation of ATP, smoking induced placental mitochondrial dysfunction can affect the nutrient transfer by the placenta, resulting in fetal stress and PTB. Our study would suggest that there are variants in the complex I mitochondrial electron transport chains that are susceptible to damage by maternal smoking and individuals with these variants are susceptible to PTB. The placental mitochondria may be where smoking, induced oxidative stress by smoking, can induce adverse effects leading to PTB.

Based on our data on three markers, we cannot conclude the extent of mtDNA contributions to PTB. However, the strong effect sizes and the trend observed suggest

that dismissing the mitochondrial genome's role in PTB would be premature. Given a larger dataset is likely to demonstrate an association with these marker and PTB. Future studies are warranted to confirm the role of mtDNA with PTB.

CHAPTER VII

PATTERNS OF CYTOKINE PROFILES DIFFER WITH PREGNANCY OUTCOME AND ETHNICITY

Overview

PTB is hypothesized to be a disease mediated by a host inflammatory response resulting from the release of cytokines. It is likely that no single factor alone adequately explains PTB risk; rather, it is more probable that coordinated networks of cytokines affect risk. Therefore, we examined the relationships between cytokines/chemokines and related biomarkers associated with PTB (cases) and normal term deliveries (controls) in African Americans and Caucasians, separately. Th1 cytokines/chemokines and related molecules interleukin (IL)-1, IL-8, and tumor necrosis factor (TNF)-α, TNF soluble receptors (sTNF-R1 and sTNF-R2) and Th2 cytokines (IL-6 and IL-10) that were previously shown to be associated with PTB were studied. Correlations between biomarkers were calculated; statistical differences between correlations of African Americans and Caucasians, and between cases and controls were measured. Multiple differences in correlations were observed between African Americans and Caucasians for both cases and controls. Correlation analyses of cytokine/chemokine measurements suggest a complex and coordinated interplay between cytokines during pregnancy and that significant differences exist in the profiles between African Americans and Caucasians, indicating that such analyses may serve as a means of understanding differential risk factors in these ethnic groups.

Introduction

Cytokine and chemokine networks function to maintain homoeostasis during pregnancy. Strong inflammatory response mediated by cytokines and chemokines are involved in pregnancy complications such as PTB. In addition, recent *in vitro* and *in vivo* findings suggest that inflammatory response mediated by cytokines and chemokines may not be the same in different geographic populations, and that this may contribute to disparity of PTB rates seen between African Americans and Caucasians in the U.S. (Demissie et al., 2001;Martin et al., 2003). It is likely that differences in pregnancy outcomes are affected by coordinated regulation of cytokines. Differences in such coordination between cytokines in African Americans and Caucasians may partially explain the disparity in PTB rates. Examining these patterns may therefore be important in understanding PTB risk and ethnic disparity.

Cytokines regulate pregnancy in an autocrine and paracrine way. Pregnancy can be seen as maternal immune adaptation to a temporary semi-allogenic graft (fetus) during gestation (Shurin et al., 1999;Thellin et al., 2000). One of the key events of a normal pregnancy is a bias towards Th2 immune response that down-regulates several cellular immune effector functions, such as cytotoxic response and inflammatory cytokine release to maintain pregnancy (Diehl and Rincon, 2002;Ugwumadu, 2002). In contrast to an upregulated Th2 profile, an imbalance of Th1/Th2 production in favor of Th1 can induce labor (Ugwumadu, 2002). Th2 cytokines predominate during early stages of pregnancy in a positive feedback loop that leads to increased production of progesterone. Progesterone in turn increases Th2 cytokine secretion (such as interleukin (IL)-10 and IL-6) and decreases Th1 cytokines (such as IL-1β, IL-8 and TNF-α), completing the positive

feedback loop that maintains pregnancy (Diehl and Rincon, 2002;Ugwumadu, 2002). At term, this balance is shifted and slowly trends in favor of Th1 cytokines that are predominantly proinflammatory. A predisposition to a premature shift in Th1-Th2 balance may elucidate risk factors and racial disparity in immune response in PTB. Such a change can be assessed by understanding the correlation structure of Th1 and Th2 cytokines in pregnancy.

Several genetic and functional studies have been performed associating both pro- and anti-inflammatory cytokines with PTB, but these studies usually analyzed cytokines individually (Dizon-Townson et al., 1997;Dudley, 1997;Menon and Fortunato, 2007;Rizzo et al., 1998;Roberts et al., 1999;Sadowsky et al., 2006;Simhan et al., 2003;Speer et al., 2006;Weiyuan and Li, 1998). These data and those indicating PTB is predominantly associated with infection (Clark and Croitoru, 2001;Dizon-Townson et al., 1997;Gardella et al., 2004;Kniss and Iams, 1998) and/or an exaggerated inflammatory response (Dudley, 1997;Goldenberg et al., 2000;Gomez et al., 1995;Kniss and Iams, 1998) that correlate with increased Th1 cytokine concentrations, have led to the hypothesis that PTB is a host inflammatory response disease. We further hypothesize that differences in patterns of cytokine production contribute to PTB pathophysiology. Hence, it is important to understand how cytokines and their receptors are regulated in preterm and term deliveries, and whether the patterns of coordination differ between African Americans and Caucasians. Differences in coordinated regulation may be indicative of different risk profiles that will be important in assessing disparity and the role of networks in disease risk.

In this Chapter we examine the correlations among several cytokines, previously associated with PTB, to assess whether there is evidence for differential cytokine production patterns between PTB (cases) and normal term deliveries (controls) and also between ethnic groups (African Americans and Caucasians) to understand ethnic disparity. Specifically, we have examined the correlations between cytokines/chemokines (IL-1β, IL-6, IL-8, IL-10, and TNF-α) and biomarkers (soluble sTNF-R1 (sTNF-R1) and soluble sTNF-R2 (sTNF-R2)). Correlations were also evaluated with respect to MIAC.

Materials and Methods

Study population

African Americans and Caucasians were used in the present study. For a description of ascertainment and recruitment please refer to Chapter III part A.

Demographic and clinical characteristics

Our study included Caucasians (105 cases and 100 controls) and African American (41 cases and 91 controls) of non-Hispanic origin. Placental and umbilical cord pathology was performed in all cases to document histologic chorioamnionitis and funisitis. The demographic and clinical characteristics for African American and Caucasian cases and controls are listed in Table 7-1.

Table 7-1. Baseline characteristics

Variable	Caucasians					African Americans				
	Controls (N=100) Median[1]		Cases (N=105) Median[1]		P-Value[2]	Controls (N=91) Median[1]		Cases (N=41) Median[1]		P-Value[2]
Maternal Age (yrs)	28[18-40]		28[18-40]		0.309	24[18-38]		25[18-38]		0.474
Gestation Age (wks)	39[37-42]		34[24-36]		<0.001	39[37-41]		34[23-36]		<0.001
Birth Weight (g)	3352[2134-4252]		2151[370-3790]		<0.001	3255[2075-4382]		2267[746-3685]		<0.001
APGAR 1	9 [4-9]		8 [1-9]		<0.001	8 [2-9]		8 [1-9]		0.012
APGAR 5	9 [8-10]		9 [1-9]		<0.001	9 [7-10]		9 [6-9]		<0.001
Smoking (Count Y=Yes, N=No)	18Y	80N	25Y	71N	0.198	13Y	77N	10Y	31N	0.165

[1]IQR-interquartile range
[2]P compares cases to controls
Non-parametric Mann-Whitney two-sample rank sum tests were used if data was not normally distributed

Cytokine and chemokine measurements

IL-1β, IL-6, IL-8, IL-10, TNF-α and sTNFR1 and sTNFR2 concentrations in the AF were measured by multiplex assay (Biosource International, Camarillo, CA), using Luminex™ (Austin, TX). The details of the assay procedure and details about the AF sample collection can be found in Chapter III part A.

Statistical methods

Shapiro-Wilks tests of normality were performed on cytokine measurements. All measurements deviated significantly from normality; as a result nonparametric tests were used for all tests of significance. Mann-Whitney two-sample rank sum tests (Hollander and Wolfe, 1999) were used to test for statistical differences of cytokines between cases and controls in each population and in cases stratified by MIAC status. STATA 9.0 statistical software (StataCorp LP, College Station, TX) (StataCorp, 2007) was used for all analyses. Correlations between all pairwise combinations of cytokine measurements were calculated for cases and controls in African Americans and Caucasians separately. Cases were also stratified by MIAC status within each ethnic group. Spearman's rho, a

non-parametric alternative to the correlation coefficient r, was used to calculate correlations using JMP statistical software (JMP Start Statistics. SAS Institute Inc., Canada) (Sall et al., 2005). Differences or heterogeneity between correlation coefficients were tested by z-test of the Fisher's r to z transformation of the Spearman rho correlation coefficient (Sokal and Rohlf, 1987). This approach has been previously published (Asselbergs et al., 2007;Reilly et al., 1994). Briefly, significant correlations are represented by lines connecting cytokines, and the strength of the correlation is represented by the thickness of each line.

Analyses were performed separately on African Americans and Caucasians. This was done because prior analyses strongly support the hypothesis that patterns of protein concentrations, as well as, the genetic regulation of these concentrations may be different between these two groups (Demissie et al., 2001;Martin et al., 2003;Menon et al., 2007).

Results

Baseline characteristics

Significant differences between cases and controls were observed in both African Americans and Caucasians for gestational age (weeks) ($p<0.001$ for both), birth weight (g) ($p<0.001$ for both), APGAR 1 (Caucasians $p<0.001$; African Americans $p=0.012$) and APGAR 5 (at 5 minutes after birth) ($p<0.001$ for both) (Table 7-1).

Cytokine measurements

Median cytokine concentrations and interquartile ranges are reported for African American and Caucasian cases and controls (Table 7-2a) and for cases with and without MIAC (Table 7-2b). Within Caucasians, there were significant differences between cases

and controls for IL-8 (p=0.002) and a marginally significant result in IL-6 (p = 0.07). There were no statistically significant differences between Caucasian cases with and without MIAC (Table 7-2b) for any of the biomarkers examined. Multiple significant differences in median analyte concentrations between cases and controls were observed in African Americans, including IL-1β (p<0.001), TNF-α (p <0.001), sTNF-R1 (p = 0.012), and sTNF-R2 (p = 0.003) (Table 7-2a). Several differences were also observed between African American cases with and without MIAC (for IL-1β, p= 0.015; for TNF-α p = 0.007; and for sTNF-R2, p = 0.015) (Table 7-2b). No differences between cases and controls were observed in either African Americans or Caucasians for IL-10.

Table 7-2. Medians and interquartile ranges and tests of median differences of cytokine concentrations

a.

Cytokine concentrations (pg/ml)	Caucasian Controls (N=100) Median [IQR][1]	Caucasian Cases (N=105) Median [IQR][1]	P	African American Controls (N=91) Median [IQR][1]	African American Cases (N=41) Median [IQR][1]	P
IL-1β	22.64[0.01-1122.67]	25.64[3.81-1008.72]	0.109	23.74[2.09-432.95]	237.7[7.00-1989.90]	<0.001
IL-8	654.3[36.27-17363.86]	1224.78[28.92- 10529.00]	0.002	729.29[12.07-15847.57]	1617.34[25.3-31302.64]	0.059
TNF-α	67.62[1.66-2493.49]	138.39[5.36-3508.92]	0.075	67.905[1.66-4476.42]	1009.34[2.09-17807.91]	<0.001
sTNF-R1	2156.89[297.34-16282.3]	2388.42[160.56-15588.60]	0.611	3249.17[297.34-12425.15]	1891.70[54.47-18126.81]	0.012
sTNF-R2	2681.47[152.5-9227.4]	2581.95[282.31-10946.19]	0.713	3294.08[723.94-10175.73]	2025.97[158.97-9581.80]	0.003
IL-6[2]	2305.71[108.42-27269.65]	3383.51[118.32-38734.73]	0.070	2396.60[160.76-19611.7]	22103.43[101.46-39712.64]	0.780
IL-10	22.33[2.16-525.02]	20.30[1.58-817.10]	0.267	12.32[0.99-3043.49]	9.57[1.58-886.21]	0.439

b.

Cytokine concentrations (pg/ml)	Caucasian No MIAC (N=79) Median [IQR][1]	Caucasian MIAC (N=25) Median [IQR] [1]	P	African American No MIAC (N=31) Median [IQR][1]	African American MIAC (N=10) Median [IQR][1]	P
IL-1β	25.64[2.26-1008.72]	28.88[4.92- 1147.63]	0.538	62.26[7.00- 1989.90]	1179.27[26.70-1760.33]	0.015
IL-8	1186.07[28.92-9876.35]	1370.23[84.93-10529]	0.697	1266.87[25.30- 31302.64]	1709.37[81.20-7325.40]	0.851
TNF-α	139.42[5.36-2080.66]	114.83[9.86- 3508.92]	0.547	373.68[2.09-8897.65]	6234.50 [209-17807.91]	0.007
sTNF-R1	2042.15[160.56-14431.83]	2942.96[354.80-15588.60]	0.151	1160.66[54.47-18126.81]	4976.43[66.56-10041.63]	0.057
sTNF-R2	2473.68[282.31-10946.19]	2955.90[392.06-10094.20]	0.498	1476.82[184.22-8379.87]	4733.60[158.97- 9581.80]	0.015
IL-6	2648.04[118.32-38734.73]	5211.63[191.49-33087.00]	0.155	3350.75[101.46-39712.64]	1987.60[407.50- 26347.34]	0.816
IL-10	21.65 [1.58-817.10]	16.56[2.16-282.17]	0.590	7.56[1.58-428.17]	37.50[1.74- 886.21]	0.101

*Non-parametric Mann-Whitney two-sample rank sum tests were used as data was not normally distributed

[1][IQR]-interquartile range

[2] Of note, in a larger sampled dataset we observed that IL-6 was statistically significant in our Caucasian population but not in our African American population and the present analysis shows that it trends toward significance. (Menon et al., 2007).

Correlations in African American and Caucasian cases and controls

Multiple significant correlations between cytokine concentrations were observed in both cases and controls (Figure 7-1; Appendix Table 5; Appendix Figure 5a and b). Approximately 39% of the Caucasian control correlations were statistically significant. In Caucasian controls, IL-6, sTNF-R1, and sTNF-R2 each had five significant positive correlations while TNF-α and IL-10 had four and IL-8 had three significant positive correlations with other biomarkers (Figure 7-1b). Of note, IL-1β had no significant correlation with any other cytokines in Caucasian term pregnancies. In Caucasian cases, approximately 36% of the correlations were statistically significant. Of these, there were five significant positive correlations between IL-8 and other cytokines and four positive correlations between IL-10 and other cytokines. Both sTNF-R1 and sTNF-R2 had three significant correlations with cytokines. Of the significant correlations, 11 were between Th1 and Th2 related biomarkers/cytokines, 9 were between Th1 related biomarkers/cytokines, and two were between Th2 related biomarkers/cytokines.

In African American controls, 47% of the correlations were statistically significant (Figure 7-1c and d; Appendix Table 5). Both sTNFR1 and sTNFR2 had four positive correlations with other cytokines, while both IL-8 and IL-10 had six, TNF-α had five, IL-6 had three and IL-1β had two (Figure 7-1d). In African American cases, 39% of the correlations were statistically significant. TNF-α, sTNF-R1, sTNF-R2, and IL-1β each had four positive correlations with other cytokines, while IL-6 and IL-8 each had two, and IL-10 had six. Of the significant correlations among African Americans 13 were between Th1 and Th2 related biomarkers/cytokines, 13 were between Th1 related biomarkers/cytokines, and two were between Th2 related biomarkers/cytokines.

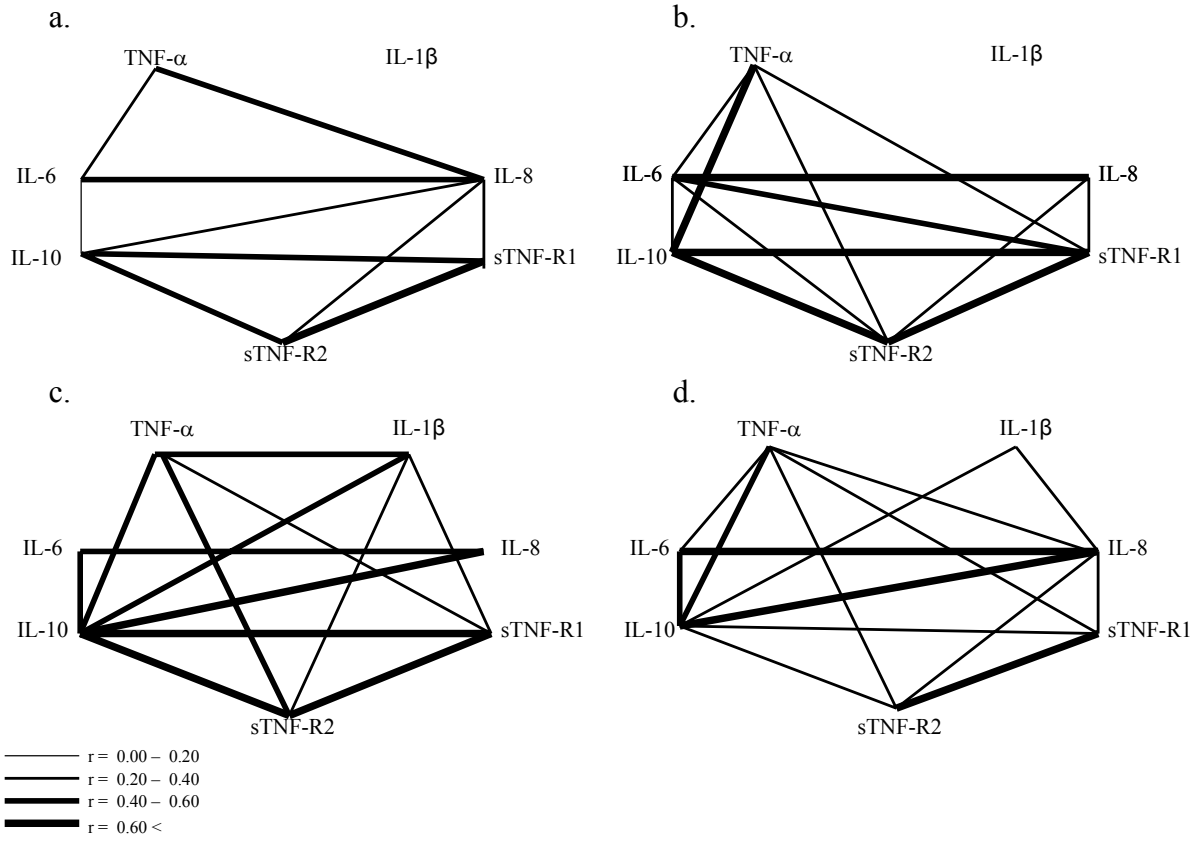


Figure 7-1. Statistically significant correlations between cytokines
a) Caucasian cases; b) Caucasian controls; c) African American cases; d) African American controls. Lines connect markers that had statistically significant correlations. Correlation (r) values are described in the legend above.

Significant correlations in cases with and without MIAC

We next examined correlations in cases with and without MIAC in order to assess whether infection affects the correlation structure (Figure 7-2a-d; Appendix Table 6). In Caucasian cases with MIAC IL-10, IL-8, and sTNF-R2 had three positive correlations with other cytokines, while sTNF-R1 had two, and IL-6 had one (Figure 7-2b). In cases without MIAC, IL-8 and IL-10 had four positive correlations with other cytokines, while IL-6 and sTNF-R2 had three, and TNF-α and sTNF-R1 had two (Figure 7-2a). Of the

significant correlations in Caucasians, three were present in cases without MIAC but not in cases with MIAC: IL-10 and IL-6, TNF-α and IL-6, and TNF-α and IL-8.

In African American cases with MIAC IL-6, sTNF-R2, IL-1β, and IL-8 had two positive correlations with other cytokines, while IL-10 had four. Of note, TNF-α had no positive or negative correlations in MIAC cases (Figure 7-2d). In African American cases without MIAC IL-8, sTNF-R1, and sTNF-R2 each had two positive correlations with other cytokines, while IL-6 had one and IL-10 had three (Figure 7-2c). Of note, IL-1β had no significant correlations in cases without MIAC as compared to the two in the presence of MIAC. Three significant correlations in African Americans were present only in cases without MIAC: TNF-α and IL-6 (-), sTNF-R1 and IL-10 (+), and sTNF-R2 and sTNF-R1 (+). Correlations present only in African American cases with MIAC are: IL-10 and IL-1β, IL-10 and IL-6, and sTNF-R2 and IL-1β.

Of the significant correlations in either Caucasians or African American cases, only one was negative regardless of MIAC status. African American cases without MIAC had a significant negative correlation for TNF-α and IL-6.

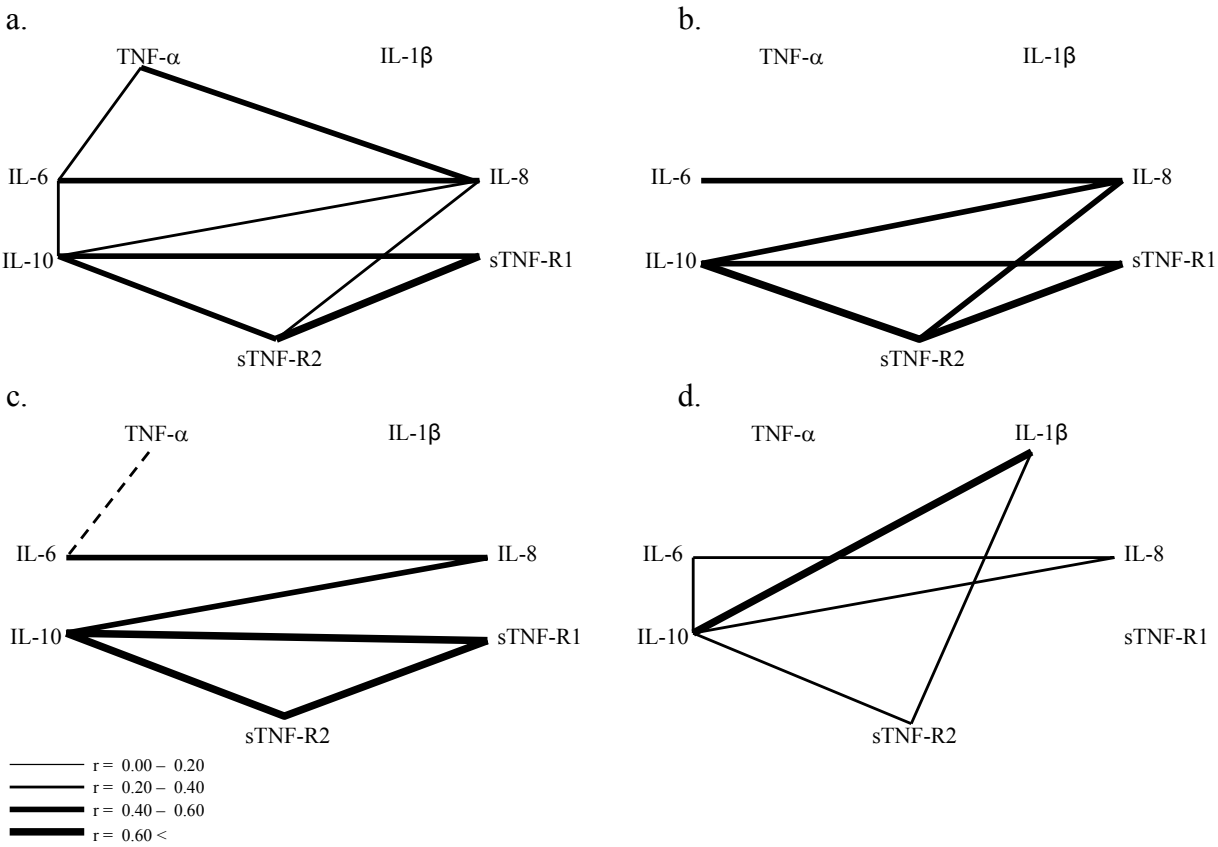


Figure 7-2. Statistically significant correlations between cytokines in cases with and without MIAC

a) Caucasians no MIAC; b) Caucasians MIAC; c) African American no MIAC; d) African American MIAC. Lines connect markers that had statistically significant correlations. Correlation (r) values are described in the legend above.

Analysis of correlation differences

Case-control comparisons within each race. To assess whether correlations differed between groups, we tested for differences between correlation coefficients (Figure 7-3a and b; Appendix Table 7). In Caucasians three comparisons were significantly different between cases and controls, one between Th1 related cytokines (TNF-α and IL-8) and two between Th1 and Th2 related analytes (TNF-α and IL-10, and IL-6 and sTNF-R1) (Figure 7-3a). In African Americans four comparisons exhibited significant correlation differences between cases and controls of which three are between

Th1-Th2 related cytokines (TNF-α and IL-6; sTNF-R1 and IL-10; and sTNF-R2 and IL-10) one between Th1 related cytokines (sTNF-R1 and IL-1β) (Figure 7-3b). Of note, two correlations switch direction between cases and controls in African American, IL-6 with TNF-α and IL-1β with sTNF-R1.

African American and Caucasian comparison within cases and controls. Substantial differences were observed for correlations between Caucasians and African Americans when comparing cases to cases or controls to controls (Figure 7-3c and d; Appendix Table 7). Eight correlations demonstrated significant differences between Caucasian and African American cases. Four were between Th1 and Th2 related cytokines (IL-10 and IL-1β, TNF-α and IL-6, TNF-α and IL10, sTNF-R2 and IL-10) and four were between Th1 related analytes (TNF-α and IL-1β, sTNF-R1 and IL-1β, sTNF-R2 and IL-1β, sTNF-R2 and TNF-α). Five correlations exhibited differences between Caucasian and African American controls: IL-10 and IL-1β, IL-10 and IL-8, sTNF-R1 and IL-6, sTNF-R1 and IL-10, sTNF-R2 and IL-10.

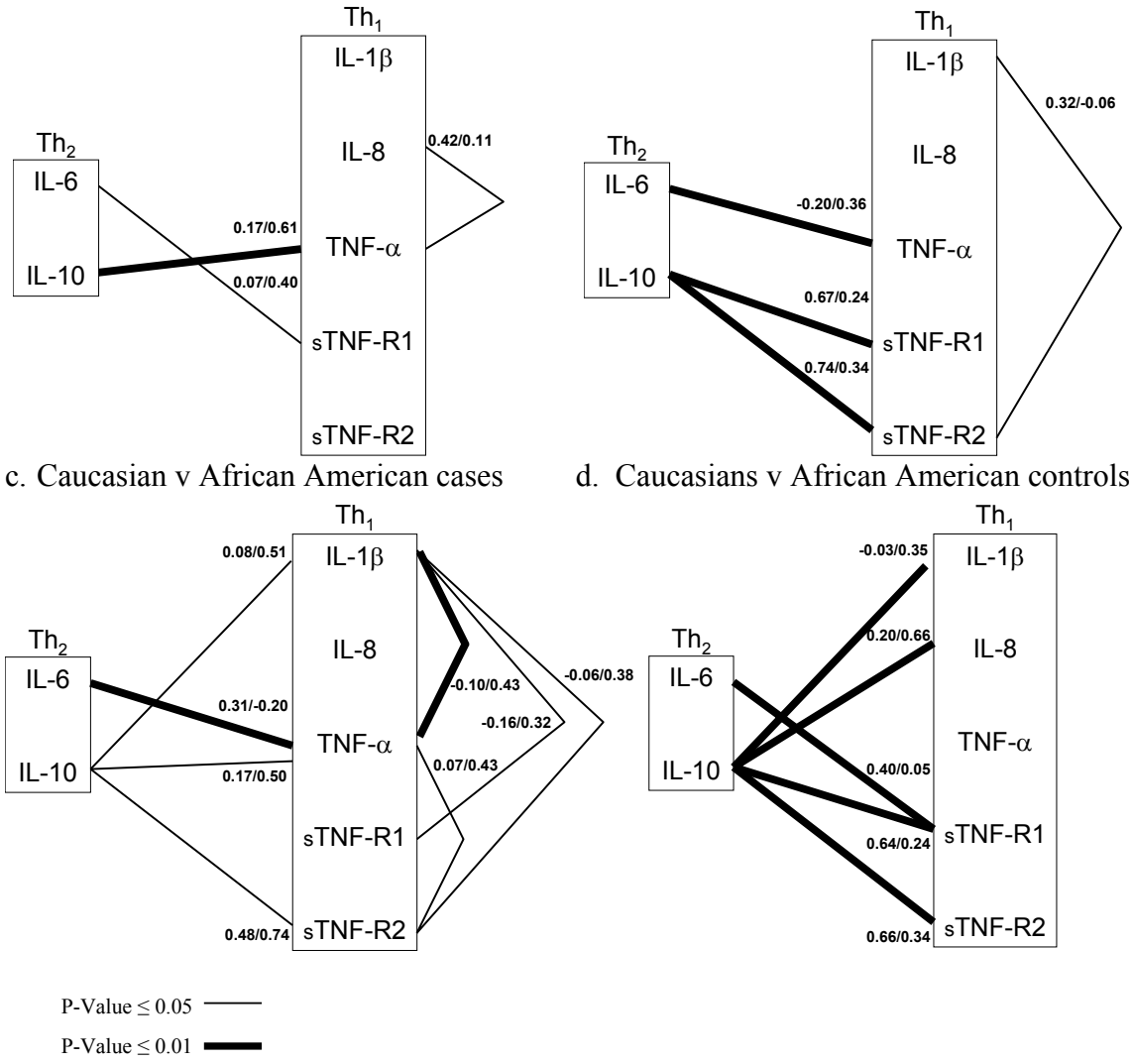


Figure 7-3. Significant correlation differences between Caucasians and African Americans and between cases and controls within a population

a) Caucasian cases versus controls ; b) African American cases versus controls; c) Caucasian versus African American cases; d) Caucasian versus African American controls. Lines connect markers that had statistically significant differences between correlations. Thick lines indicate a significance $p \leq 0.01$ and thinner lines indicate correlations with $p \leq 0.05$.

Significant correlation differences for cases with and without MIAC. There were no statistically significant correlation differences between Caucasian cases with and without MIAC (Appendix Table 8). In Caucasians, correlations between cases and

controls that showed significant correlation differences demonstrated no such effect between cases with and without MIAC. However, in African Americans three correlations showed correlation differences between cases with and without MIAC: IL-10 with IL-1β ($p = 0.004$; no MIAC $r = 0.35$, MIAC $r = 0.91$); TNF-α with IL-6 ($p = 0.004$; no MIAC $r = -0.43$, MIAC $r = 0.64$); sTNF-R2 with IL-1β ($p = 0.039$; no MIAC $r = 0.11$, MIAC $r = 0.75$).

Discussion

The role of cytokine production patterns in PTB and in two ethnic groups were examined by assessing correlation structures in control vs. case amniotic fluids, in both Caucasians and African Americans. The goal of this analysis was to better elucidate the role of cytokine networks in PTB. Our data revealed significant differences between correlations in African Americans and Caucasians in both cases and controls, revealing disparity in immune response. In addition, within an ethnic group we found several differences between cases and controls. Our Th1 and Th2 grouping should be tentatively considered given that these cytokines were selected for being PTB candidates and not for Th1/Th2 grouping. Nonetheless, interesting patterns were observed that provides some indications of Th1 and Th2 effects, and that merit follow-up in future studies with more classically accepted Th1/Th2 cytokines.

Within Caucasians, the strongest evidence of differences in correlation between cases and controls was between TNF-α and IL-10. The differential coordination of IL-10 and TNF-α is of potential significance because IL-10 is a regulator of TNF-α and its production is known to be significantly reduced in the placenta of patients at term

compared to first and second-trimester tissues (Hanna et al., 2000;Romero et al., 2007). Based on previous studies of decidual leukocytes TNF-α and IL-10 concentrations are highly correlated during normal labor, with correlations of ~0.6 (Gustafsson et al., 2006), indicating a balanced immune status between pro and anti inflammatory cytokines. We observed results consistent with these previous reports in controls of both Caucasians and African Americans, but this correlation is not observed in cases in either ethnic group. These results may indicate a trend towards immune imbalance in cases.

In African Americans significant differences between correlation coefficients were observed between IL-6 and TNF-α (-0.20 in cases and 0.36 in controls). In controls, the two cytokine concentrations were positively correlated, suggesting a generalized inflammatory status during labor. However, the correlation coefficient is negative in cases with the increase in TNF-α not being coordinated with the IL-6 concentrations, indicating an overwhelming TNF-α response.

Another intriguing result is for IL-1β. In case-case comparisons, correlation coefficients indicated that African Americans and Caucasians differed significantly for IL-1β/TNF-α and sTNF-R1/sTNF-R2 correlations. These results suggest that the IL-1β is coordinately co-regulated with several other cytokines in African American cases; however, this co-regulation is absent in Caucasians, again indicating a difference in the underlying processes leading to PTB in different ethnic groups. Recent animal model studies documented that IL-1β and TNF-α are inducers of preterm labor whereas IL-6 and IL-8 failed to induce labor even after prolonged administration, although both IL-6 and IL-8 produced inflammatory changes in the fetal membranes and lungs. Our data

support these findings in African Americans where preterm labor may be mediated predominantly by TNF-α and IL-1β (Sadowsky et al., 2003).

Correlation differences were observed between immune function regulators in African Americans. IL-10 was significantly more correlated with sTNFR1 and sTNFR2 in cases than controls, supporting the existence of immunoinhibitory mechanisms during preterm birth. However, a proinflammatory surge, as seen by increases in TNF-α and IL-1β, may overwhelm the inhibitors further supporting our claim that PTB is a host inflammatory disease.

The potential role of infection in African American compared to Caucasian pregnancies is also illustrated by evidence for differences between correlations in cases with and without MIAC in the two groups. We emphasize that the definition of MIAC was not based solely on the detection of bacterial DNA in the AF but also clinical evidence of MIAC that correlate with PCR data. However, it is possible that we could not detect presence of microbes between membranes or in biofilms in the AF. We did not find strong evidence for correlation differences in Caucasian cases with and without MIAC. This suggests the coordinated cytokine activity in Caucasians cases is not significantly affected by the presence of infection. Although MIAC may affect individual cytokine concentrations in presence of certain genetic predispositions in Caucasians (Velez et al., 2007a), infection alone does not appear to explain the observed heterogeneity between Caucasians case-control cytokine correlations. In contrast in African American cases, there was evidence for heterogeneity between MIAC and no MIAC, although these differences were observed in only 14% of our comparisons (3/21). We caution, however, that these secondary analyses may not be adequately powered to

detect effects of biological importance and that our results with respect to MIAC are preliminary.

It is important to note that the current analysis is different from previous ones in that we are not explicitly comparing changes in protein levels and/or cytokine ratios, but the relationships of these within a status group. Therefore, it is possible to observe significant differences in concentrations and/or ratios, but not identify significant correlations as we have done here. This can be intimated from the current analysis by comparing thickness of lines between cases and controls in each group as well as the changes in the overall patterns. Such findings suggest, for example, that even though the ratios are different between cases and controls, two factors are both behaving monotonically. This may be interpreted as both concentrations increase but that the rate of the increase is different. We suggest that the current analyses may add to our overall understanding of how a complex set of factors can affect disease risk.

Our data provide preliminary evidence that Caucasians and African Americans have strong differences in patterns of cytokine and cytokine related biomarker production. This is especially true in cases, supporting that the observed PTB rate differences may in fact be due to differences in the underlying etiology. From the present study we cannot conclude whether the mother or fetus is contributing to our AF pool of cytokines, given that a response can be initiated by both mother and the fetus and measurements of cytokines from the AF are a net affect of the two. Finally, in assessing patterns of coordinated cytokine production it appears that controls in the two populations have fewer correlation differences than cases of the two (Figures 7-2 and 7-3). Based on our findings we hypothesize that the pathways leading to PTB operate differently on

mediators in each ethnic group, culminating in a comparable terminal events leading to labor and delivery.

CHATPER VIII

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions and Summaries

Studies examining reproductive disorders have been poorly addressed in the human genetics literature. Most of these disorders, including PTB, do not follow a simple Mendelian mode of inheritance. In addition, PTB is unique among genetic disorders in that its etiology has both maternal and fetal biological components. Despite this obvious complexity the majority of the genetic studies on PTB performed over the last few years only consider relatively simple designs (single gene or single SNP). The studies presented in this dissertation address many of the complexities in the field and therefore, provide new insight into the genetic risks underlying PTB.

In the studies presented in the previous chapters, several interesting findings regarding the genetics of PTB have emerged. Our initial studies focused on understanding the immunological risk of PTB and how genetic variants affect risk. We did this by examining the association of IL-6 AF levels and variants in IL-6 and IL-6R genes and by examining the correlation networks between cytokines in the AF of preterm and term women. We did not replicate an association previously found in the IL-6 gene at marker C-174G. However, upon examination of IL-6 AF, we observed an association at G-661A with IL-6 AF levels in Caucasians, a marker in tight LD with C-174G. Subsequent analyses revealed that IL-6 AF levels were also significantly associated with IL-6 and even more strongly associated with IL-6R haplotypes. The regions associated

were consistent across racial populations confirming findings from previous studies that found that IL-6 protein expression levels was more strongly associated with IL-6R than with IL-6 gene variants and that a haplotype in the IL-6 promoter region has a strong regulatory role over IL-6 expression. AF correlation network analyses assessing concentrations of IL-6 and six additional cytokines revealed dramatically different correlation networks both between African Americans and Caucasians and between cases and controls. These analyses indicate that at a baseline level (term pregnancy women) the relationships between cytokines are drastically different between racial groups and these differences may explain some of the observed differences in PTB rates.

These analyses were followed up by a high-throughput study examining genes from all four hypothesized PTB pathways and a study of complex I markers of the mitochondrial genome. To our knowledge this was the first candidate gene association study comprehensively examining allelic, genotypic, haplotype, and gene-gene associations with candidate genes from all four hypothesized PTB pathways. We are also the first, to our knowledge, to extend the search to the mitochondrial genome.

Although we did not find a strong association with mtDNA markers, we did find a marginally significant association with a marker in complex I haplogroup T after stratifying by smoking status. This marker had a strong OR with borderline levels of significance that support it being followed up in a larger powered study.

The candidate gene screen across hypothesized pathways revealed several interesting results with some overlap across African American, Caucasian, maternal, and fetal populations (Figure 8-1). In all four populations pathway analyses revealed an excess of significant associations in the pathways involving cytokines. The strongest

overlap across populations (African American, Caucasian, maternal, and fetal; Figure 8-1, region I) was in genes involved in immune response pathways and decidual hemorrhage. The most promising association in these studies was seen in tPA and pathways involving tPA in the maternal Caucasian samples.

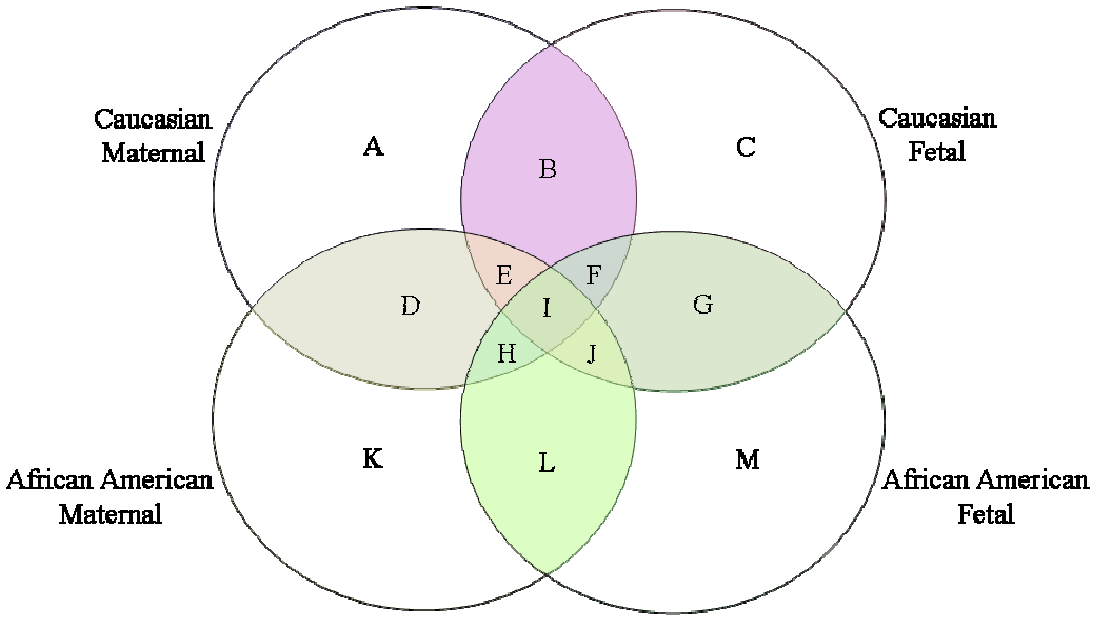


Figure 8-1. Overview of overlapping associations across populations.

Gene included in each region: **A**-COL1A1, DHFR, FVII, HSD11B1, IL-18, PGRMC1, and TLR2; **B**-COL1A2, CYP19A1, CYP2D6, UGT1A1, IL-5, MMP1, NFKB1A, and tPA; **C**-CBS, CRHR2, GSTP1, HSPA14, IGF1, IL-1A, PGR, PTGS2, and SLC23A1; **D**-CCL2, IL-6-R, PLA2G4A, and TLR7; **E**-IL-4-R; **F**-CRHBP, MMP3, and NFKBIB; **G**-EDN2, NFKBIE, and TREM1; **H**-COL5A1, CTLA4, FV, IL-1R1, sTNF-R1, sTNF-R2, and VEGF; **I**-COL3A1, COL5A2, EPHX2, IL-10RA, IL-1RAP, IL-2RA, KL, PON2, PTGER3, TIMP3, and TSHR;**J**-IL-1RN, IL-2-RB, IL-4, and MMP8; **K**-ACE, ADRB2, CCL8, HSD17B7, HSPA1B, HSPA1L, HSPA4, PGRMC2, POMC, and TLR8; **L**-CARD15, EPHX1, IFNG, IL-10-RB, IL-15, IL-6, IL-8, MMP2, MMP9, NFKB1, PGEA1, and SLC6A4.

Several associations were found in the compliment and coagulation pathway in Caucasian maternal samples. As described in Chapter IV part A, the compliment and coagulation pathway yielded several significant associations, including an excess of significant single locus associations within this pathway. Associations were seen with FV, FVII, and tPA at the allele and/or genotype level. A statistically significant interactive effect was also observed between FV and FVII when tPA was excluded,

indicating that heterogeneity within the compliment and coagulation pathway may contribute to susceptibility to PTB in Caucasians.

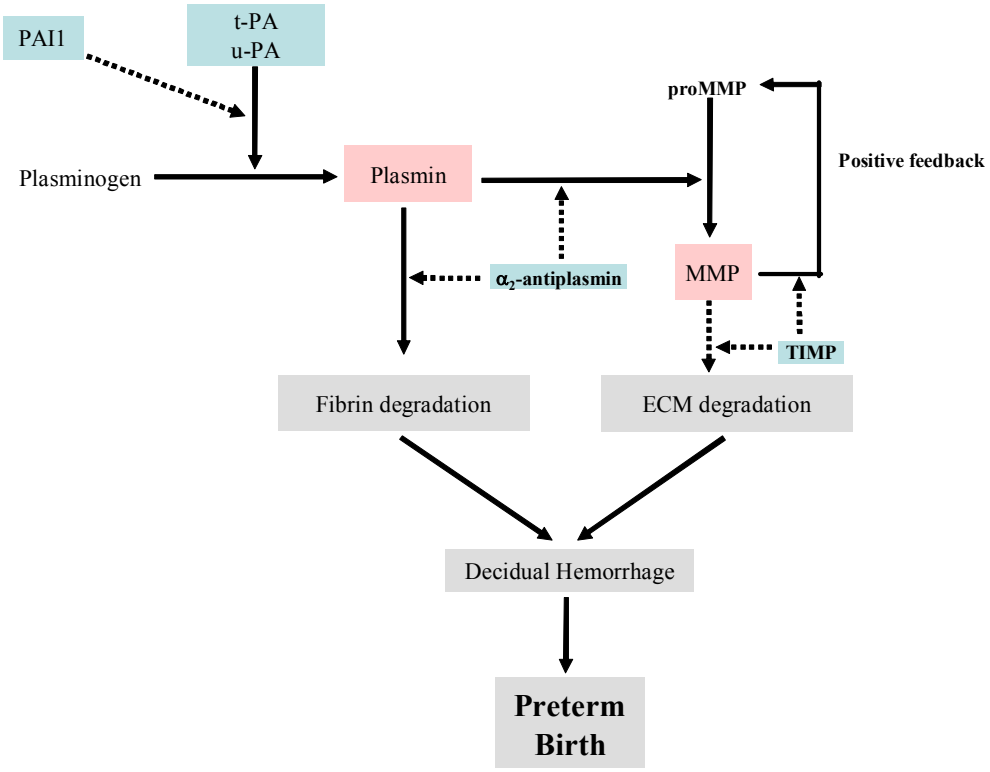


Figure 8-2. Proposed pathway for tPA in spontaneous preterm birth.

Figure 8-2 shows a potential mechanism by which mutations in the compliment and coagulation pathway may lead to PTB. A mutation in tPA or other components of the compliment and coagulation cascade can activate the conversion of plasminogen to plasmin. Activating tPA production in the process of fibrin degradation leads to small fibrin clot formation, depletion of coagulation factors, and finally hemorrhage of the decidua. Excess tPA can also increase the conversion of proMMPs with broad substrate specificity to the active secreted (zymogenic) form of MMPs. Active MMPs lead to the degradation of the extra cellular matrix (ECM) by breaking down interstitial collagens

that can compromise the structural integrity of the decidua and lead to decidual hemorrhage. Fibrin degradation may also lead to ECM degradation. Another potential mechanism by which tPA may lead to PTB is through maternal-fetal stress response pathways, this mechanism is described in the discussion of Chapter IV part A.

In conclusion, my studies identified several genetic variants in tPA, FV, FVII, CRHBP, IL-6, and IL-6R that can influence the risk of a PTB through multiple pathways. Most of the genetics studies in the field of PTB have been composed of small sample sizes, often of pooled racial grouping, and poorly defined clinical phenotypes. Our dataset was adequately powered and consisted of a clear and clinically well defined case-control retrospective cohort for all of these analyses. Unlike many other studies in the field, we excluded on several factors for our case and control definitions in efforts to avoid phenotypic heterogeneity in our analyses. This ultimately reduced our power; however, despite this we were able to find several interesting associations and are confident in our results. These results support the hypothesis that genetic factors influence the susceptibility of delivery preterm.

Future Directions

The future goals of this work are to more fully assess the significance of these findings by obtaining independent replication datasets. Given the apparent genetic heterogeneity observed in our results and the well established phenotypic heterogeneity of PTB, an ideal replication dataset will consist of a larger sample size with a homogenous population and a precise clinical definition of PTB. We anticipate having access to several replication datasets.

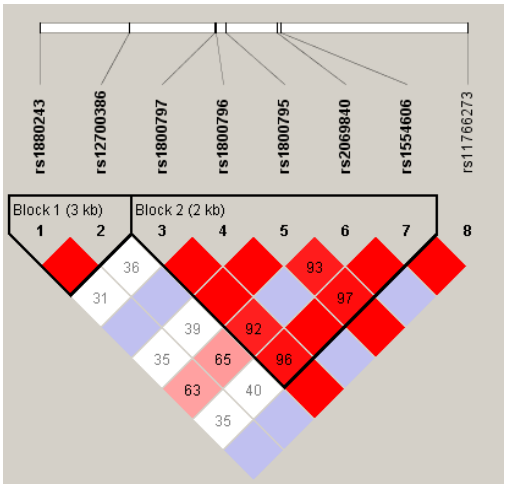
The compliment and coagulation pathway was found to have several interesting associations in Caucasian samples and the cytokine pathway was found to have interesting associations in Caucasians and African Americans, as a result we also plan on following up these pathways with more saturated analyses. We will genotype more markers within interesting genes, as well as markers in other genes within these pathways; this will allow us to assess the degree of heterogeneity occurring in these pathways. We may also follow up the genes with the strongest associations with deep resequencing.

If our findings are found to replicate, biological relevance may be further assessed by using transgenic animal models and by pursuing family-based linkage and/or association analyses, as these would allow a better understanding of the heritability of these variants. An appropriate transgenic animal model, however, may be difficult to find given that pregnancy in humans is very different relative to commonly used animal models.

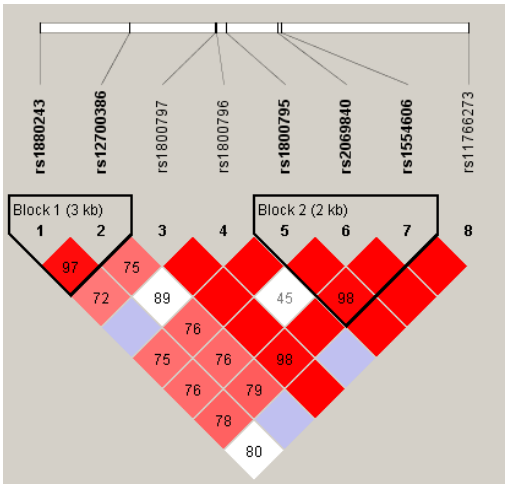
In conclusion, we identified several compelling associations with strong and diverse genetic effects. These results both support the hypothesis that PTB has a significant genetic basis and highlight that the mechanisms underlying PTB have not been fully explained by the literature and are likely to represent a series of heterogeneous risks.

APPENDIX

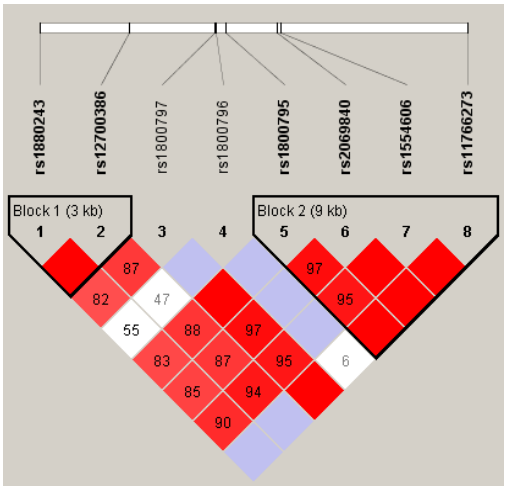
a. Caucasian maternal cases



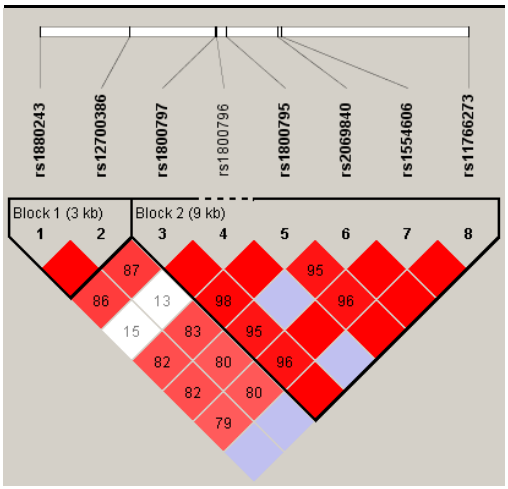
b. Caucasian maternal controls



c. Caucasian fetal cases

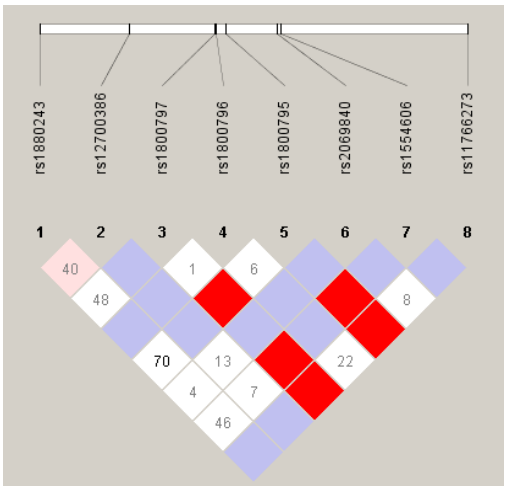


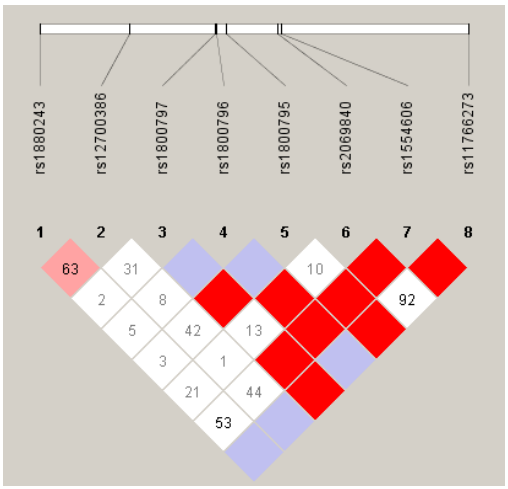
d. Caucasian fetal controls



e. African American maternal cases

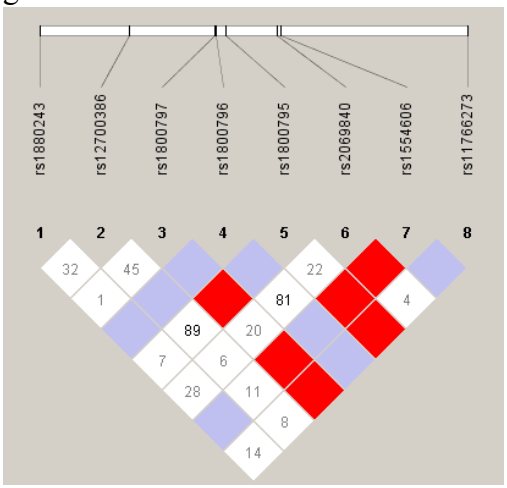
f. African American maternal controls

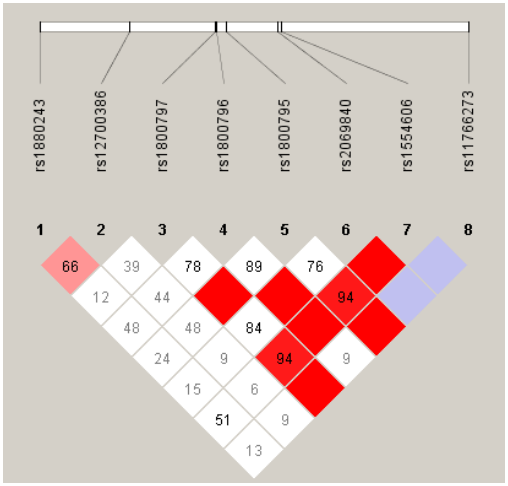




g. African American fetal cases

h. African American fetal controls

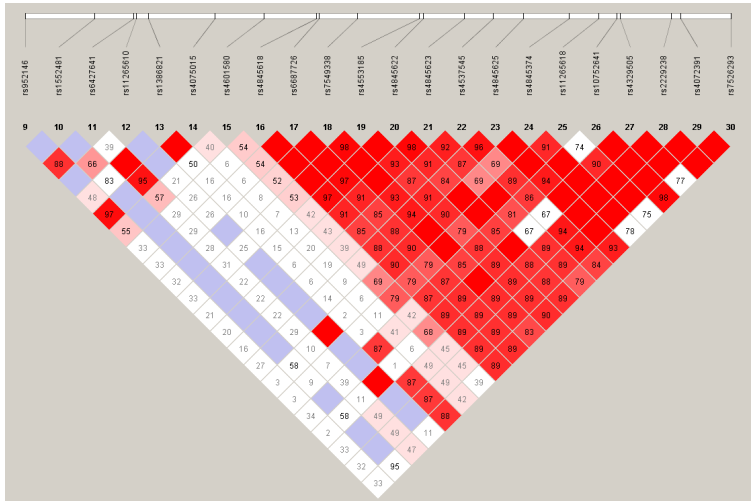




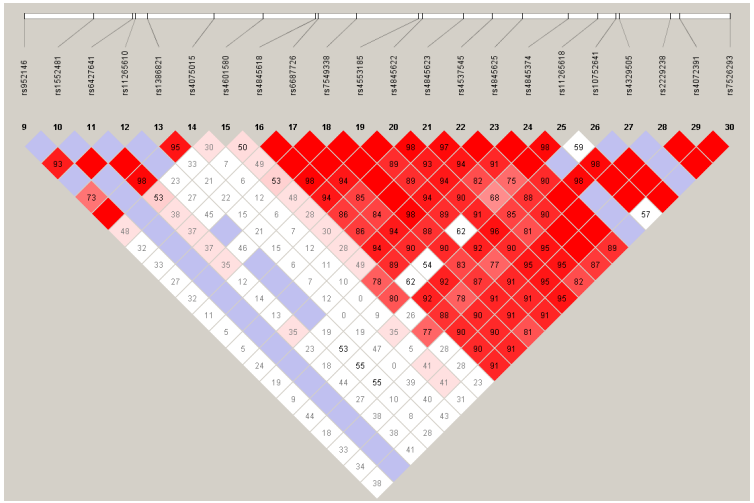
Appendix Figure 1. IL-6 haplotype block structure.

a) Caucasians maternal cases; b) Caucasians maternal controls; c)Caucasian fetal cases; d) Caucasians fetal controls; e) African American maternal cases; f) African American maternal controls; g) African American fetal cases; h) African American fetal controls. D' values are labeled within each square.

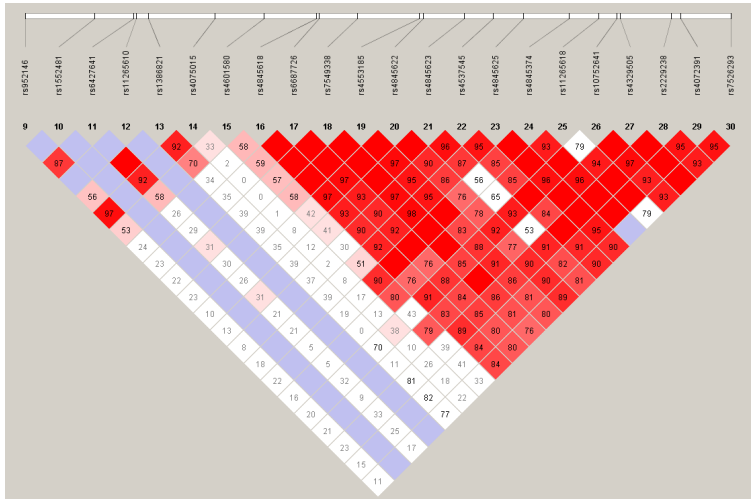
a. Caucasian maternal cases



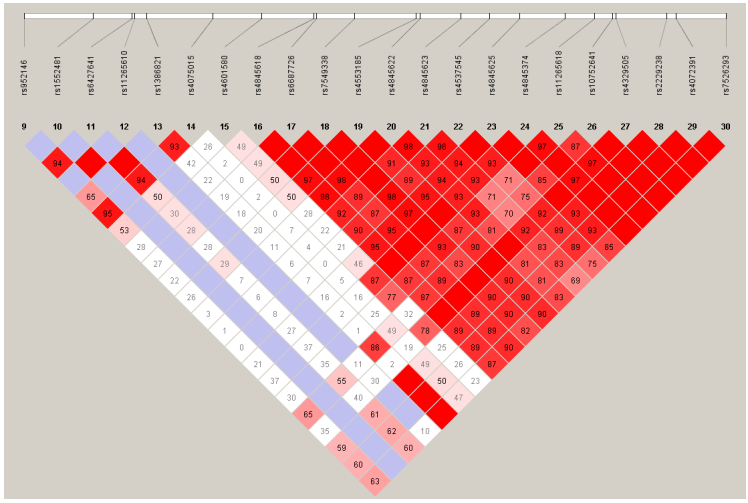
b. Caucasian maternal controls



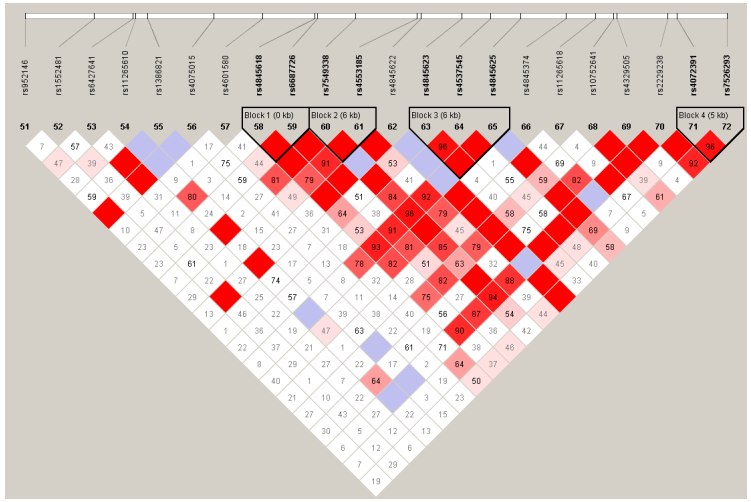
c. Caucasian fetal cases



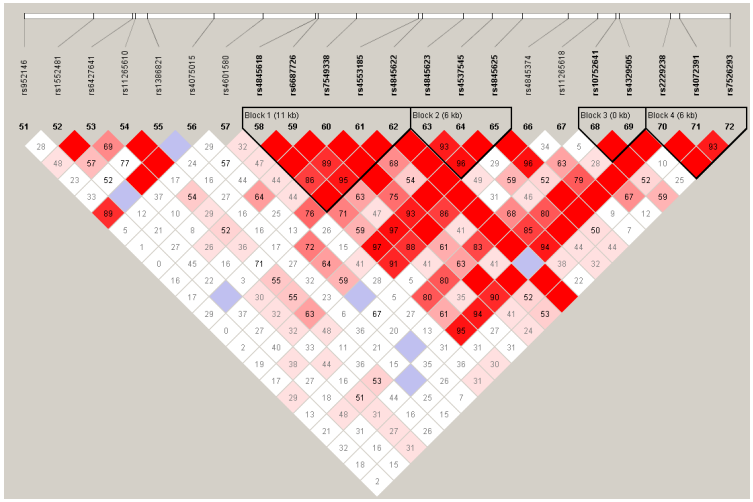
d. Caucasian fetal controls



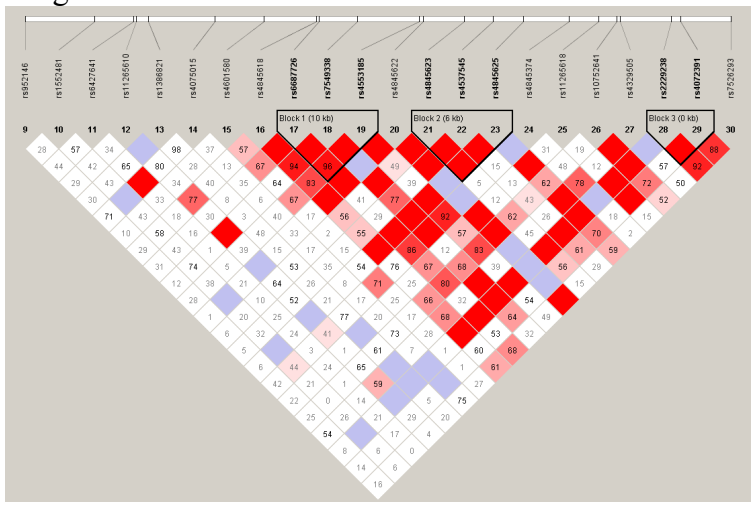
c. African American maternal cases



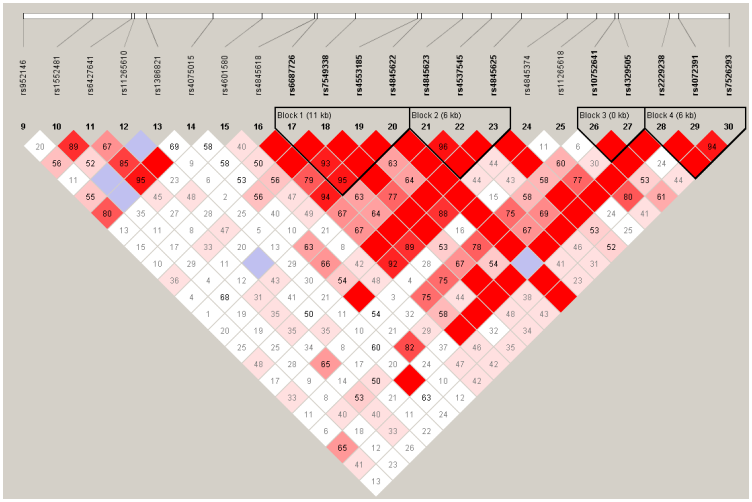
f. African American maternal controls



g. African American fetal cases



h. African American fetal controls



Appendix Figure 2. IL-6R haplotype block structure.

a) Caucasians maternal PTB; b) Caucasians maternal term; c) Caucasians fetal PTB; d) Caucasians fetal term; e) African American maternal PTB; f) African American maternal term; g) African American fetal PTB; h) African American fetal term. D' values are labeled within each square.

Appendix Table 1. Genes and number of markers genotyped in Illumina genotyping

Gene	# Markers
ACE angiotensin I converting enzyme isoform 1	9
ADH1B alcohol dehydrogenase 1B (class I), beta	11
ADH1C class I alcohol dehydrogenase, gamma subunit	6
ADRB2 adrenergic, beta-2-, receptor, surface	4
CARD15 NOD2 protein	11
CBS cystathionine-beta-synthase	16
CCL2 small inducible cytokine A2 precursor	5
CCL3 chemokine (C-C motif) ligand 3	4
CCL8 small inducible cytokine A8 precursor	7
CD14 CD14 antigen precursor	3
COL1A1 alpha 1 type I collagen preproprotein	10
COL1A2 alpha 2 type I collagen	35
COL3A1 procollagen, type III, alpha 1	21
COL5A1 alpha 1 type V collagen preproprotein	28
COL5A2 alpha 2 type V collagen preproprotein	15
CRH corticotropin releasing hormone precursor	6
CRHBP corticotropin releasing hormone binding protein	5
CRHR1 corticotropin releasing hormone receptor 1	2
CRHR2 corticotropin releasing hormone receptor 2	12
CRP C-reactive protein, pentraxin-related	3
CTLA4 cytotoxic T-lymphocyte-associated protein 4	9
CYP19A1 cytochrome P450, family 19	50
CYP1A1 cytochrome P450, family 1, subfamily A,	2
CYP2D6 cytochrome P450, family 2, subfamily D,	2
DHFR dihydrofolate reductase	6
EDN2 endothelin 2	10
EPHX1 epoxide hydrolase 1, microsomal (xenobiotic)	12
EPHX2 epoxide hydrolase 2, cytoplasmic	17
FII coagulation factor II precursor	2
FV coagulation factor V precursor	26
FVII coagulation factor VII precursor, isoform a	4
FAS tumor necrosis factor receptor superfamily,	15
FASLG fas ligand	6
GSTP1 glutathione transferase	1
GSTT2 glutathione S-transferase theta 2	1
HSD11B1 11-beta-hydroxysteroid dehydrogenase 1	13
HSD17B7 hydroxysteroid (17-beta) dehydrogenase 7	6
HSPA14 heat shock 70kDa protein 14 isoform 2	7
HSPA1A heat shock 70kDa protein 1A	3
HSPA1B heatshock 70kDa protein 1B	2
HSPA1L heat shock 70kDa protein 1-like	2
HSPA4 heat shock 70kDa protein 4 isoform a	5
HSPA6 heat shock 70kDa protein 6 (HSP70B')	6
IFNG interferon, gamma	5
IGF1 insulin-like growth factor 1 (somatomedin C)	1
IGFBP3 insulin-like growth factor binding protein 3	1
IL10 interleukin 10 precursor	5

IL-10RA interleukin 10 receptor, alpha precursor	11
IL-10RB interleukin 10 receptor, beta precursor	15
IL-13 interleukin 13 precursor	6
IL-15 interleukin 15 preproprotein	12
IL-18 interleukin 18 proprotein	8
IL-1A interleukin 1, alpha proprotein	3
IL-1B interleukin 1, beta proprotein	7
IL-1R1 interleukin 1 receptor, type I precursor	16
IL-1R2 interleukin 1 receptor, type II precursor	23
IL-1RAP interleukin 1 receptor accessory protein isoform	65
IL-1RN interleukin 1 receptor antagonist isoform 3	14
IL-2 interleukin 2 precursor	7
IL-2RA interleukin 2 receptor, alpha chain precursor	33
IL-2RB interleukin 2 receptor beta precursor	25
IL-4 interleukin 4 isoform 1 precursor	8
IL-4R interleukin 4 receptor alpha chain isoform b	27
IL-5 interleukin 5 precursor	5
IL-6 interleukin 6 (interferon, beta 2)	8
IL-6R interleukin 6 receptor isoform 1 precursor	22
IL-8 interleukin 8 precursor	3
IL-8RA interleukin 8 receptor alpha	4
KL klotho isoform a	19
LST1 leukocyte specific transcript 1 isoform 1	1
MBL2 soluble mannose-binding lectin precursor	11
MMP1 matrix metalloproteinase 1 preproprotein	12
MMP2 matrix metalloproteinase 2 preproprotein	21
MMP3 matrix metalloproteinase 3 preproprotein	5
MMP8 matrix metalloproteinase 8 preproprotein	8
MMP9 matrix metalloproteinase 9 preproprotein	4
MTHFD1 methylenetetrahydrofolate dehydrogenase 1	13
MTHFR 5,10-methylenetetrahydrofolate reductase	15
NAT1 N-acetyltransferase 1	10
NAT2 arylamide acetylase 2	7
NFKB1 nuclear factor kappa-B, subunit 1	19
NFKB2 nuclear factor of kappa light polypeptide gene	3
NFKBIA nuclear factor of kappa light polypeptide gene	3
NFKBIB nuclear factor of kappa light polypeptide gene	8
NFKBIE nuclear factor of kappa light polypeptide gene	5
NOS3 nitric oxide synthase 3 (endothelial cell)	7
NR3C1 nuclear receptor subfamily 3, group C, member 1	18
PAFAH1B1 platelet-activating factor acetylhydrolase,	12
PAFAH1B2 platelet-activating factor acetylhydrolase,	2
PGEA1 PKD2 interactor, golgi and endoplasmic reticulum	4
PGR progesterone receptor	21
PGRMC1 progesterone receptor membrane component 1	2
PGRMC2 progesterone membrane binding protein	5
PLA2G4A phospholipase A2, group IVA	30
tPA tissue plasminogen activator	8
POMC proopiomelanocortin preproprotein	1
PON1 paraoxonase 1	25

PON2 paraoxonase 2 isoform 1	14
PTCRA pre T-cell antigen receptor alpha	5
PTGER2 prostaglandin E receptor 2 (subtype EP2), 53kDa	7
PTGER3 prostaglandin E receptor 3, subtype EP3 isoform	53
PTGES prostaglandin E synthase	7
PTGFR prostaglandin F receptor isoform a precursor	12
PTGS1 prostaglandin-endoperoxide synthase 1 isoform 1	17
PTGS2 prostaglandin-endoperoxide synthase 2 precursor	7
PTPN22 protein tyrosine phosphatase, non-receptor type	1
SCNNIA sodium channel, non-voltage gated 1 alpha	1
SCGB1A1 secretoglobin, family 1A, member 1	3
SERPINE1 plasminogen activator inhibitor-1	7
SERPINH1 serine (or cysteine) proteinase inhibitor, clade	3
SLC23A1 solute carrier family 23 (nucleobase	3
SLC6A4 solute carrier family 6 member 4	14
TCN2 transcobalamin II precursor	1
TGFB1 transforming growth factor, beta 1	3
TIMP3 tissue inhibitor of metalloproteinase 3	32
TIMP4 tissue inhibitor of metalloproteinase 4	4
TLR2 toll-like receptor 2	4
TLR3 toll-like receptor 3	10
TLR4 toll-like receptor 4 precursor	13
TLR7 toll-like receptor 7	15
TLR8 toll-like receptor 8 isoform 1	15
TLR9 toll-like receptor 9 isoform B	1
TNF tumor necrosis factor alpha	6
sTNF-R1 soluble tumor necrosis factor receptor 1	9
sTNF-R2 soluble tumor necrosis factor receptor 2	19
TRAF2 TNF receptor-associated factor 2	3
TREM1 triggering receptor expressed on myeloid cells	11
TSHR thyroid stimulating hormone receptor isoform 2	63
UGT1A1 UDP glycosyltransferase 1 family, polypeptide A1	18
VEGF vascular endothelial growth factor isoform a	8

Appendix Table 2. Gene list with rs numbers

Gene	SNP rs#
ACE	rs7214530
ACE	rs4295
ACE	rs4305
ACE	rs4311
ACE	rs4362
ACE	rs4461142
ACE	rs4267385
ACE	rs4459610
ACE	rs12451328
ADH1B	rs12507573
ADH1B	rs1042026
ADH1B	rs17033
ADH1B	rs13133908
ADH1B	rs1789882
ADH1B	rs1693457
ADH1B	rs4147536
ADH1B	rs1353621
ADH1B	rs1159918
ADH1B	rs6810842
ADH1B	rs1229982
ADH1C	rs1229980
ADH1C	rs1614972
ADH1C	rs904096
ADH1C	rs3762896
ADH1C	rs17586163
ADH1C	rs1662037
ADRB2	rs1432622
ADRB2	rs12654778
ADRB2	rs1042713
ADRB2	rs4705271
CARD15	rs4785224
CARD15	rs2067085
CARD15	rs17312836
CARD15	rs2066843
CARD15	rs2066844
CARD15	rs751271
CARD15	rs2066845
CARD15	rs5743289
CARD15	rs5743291
CARD15	rs7203344
CARD15	rs8056611
CBS	rs706208
CBS	rs1051319
CBS	rs3788050
CBS	rs2124459
CBS	rs8132811
CBS	rs1005584
CBS	rs6586282

CBS	rs6586283
CBS	rs11203172
CBS	rs12329764
CBS	rs234705
CBS	rs234709
CBS	rs2851391
CBS	rs234715
CBS	rs11701048
CBS	rs1788484
CCL2	rs1024611
CCL2	rs1024610
CCL2	rs3760396
CCL2	rs4586
CCL2	rs991804
CCL3	rs1634491
CCL3	rs1851503
CCL3	rs9972960
CCL3	rs1634502
CCL8	rs885691
CCL8	rs1233650
CCL8	rs3138034
CCL8	rs3138035
CCL8	rs11575060
CCL8	rs3138039
CCL8	rs4794999
CD14	rs4914
CD14	rs2569190
CD14	rs2569193
COL1A1	rs2586482
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NAT1	rs8190837
NAT1	rs8190870
NAT2	rs4646246
NAT2	rs7832071
NAT2	rs1801280
NAT2	rs1799929
NAT2	rs1208
NAT2	rs721398
NAT2	rs721399
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NFKB2	rs7897947
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PGR	rs542384
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PLA2G4A	rs6683515
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SLC23A1	rs6596473
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TIMP4	rs4684841
TIMP4	rs3755724
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VEGF	rs699947
VEGF	rs833068
VEGF	rs833069
VEGF	rs3025010
VEGF	rs3025033
VEGF	rs3025035
VEGF	rs998584
VEGF	rs6900017

Appendix Table 3. Genotype model p values after adjusting for smoking and gravidity

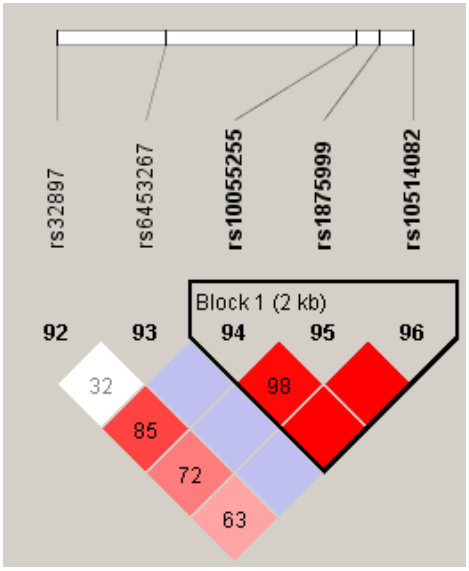
Population	*Gene(s)*	SNP rs#	Adjusted Model P[1]
Maternal	*CRHBP*	rs1875999	0.02
		rs32897	5.00×10^{-3}
		rs10055255	6.00×10^{-3}
	FV	rs9332624	-
	IL-5	rs739718	0.08
	tPA	rs879293	$<1.00 \times 10^{-3}$
	PTGER3	rs977214	0.14
		rs594454	3.00×10^{-3}
	SCNN1A	rs3764874	0.03
Infant	CBS	rs12329764	0.27
	IL-10RA	rs17121510	0.29
	KL	rs9527025	0.14
		rs522796	0.04
	TREM1	rs6910730	0.20

[1]P value for model in Table 5 adjusted smoking and gravidity

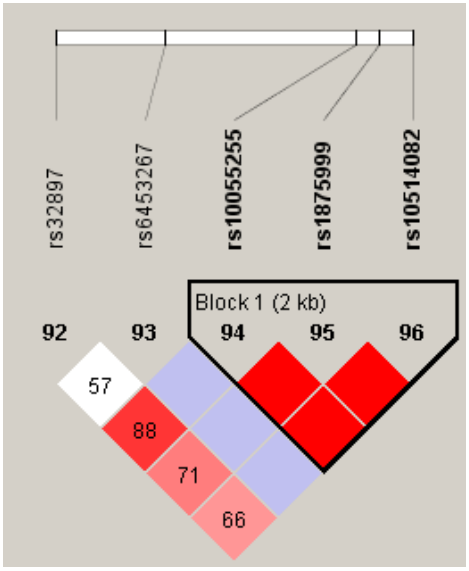
Appendix Table 4. Examining cases for allelic and genotypic differences between cases with and without MIAC

Population	*Gene(s)*	SNP rs#	Cases MIAC v PTB No MIAC	
			Allele P	Genotype P
Maternal	*CRHBP*	rs1875999	0.57	0.39
		rs32897	0.54	0.55
		rs10055255	0.42	0.73
	FV	rs9332624	0.33	0.68
	IL-5	rs739718	1.00	1.00
	tPA	rs879293	0.19	0.51
	PTGER3	rs977214	0.19	0.47
		rs594454	0.74	0.36
	SCNN1A	rs3764874	0.81	0.95
Infant	CBS	rs12329764	0.43	0.76
	IL-10RA	rs17121510	0.87	0.86
	KL	rs9527025	1.00	0.20
		rs522796	0.17	0.06
	TREM1	rs6910730	0.02	0.05

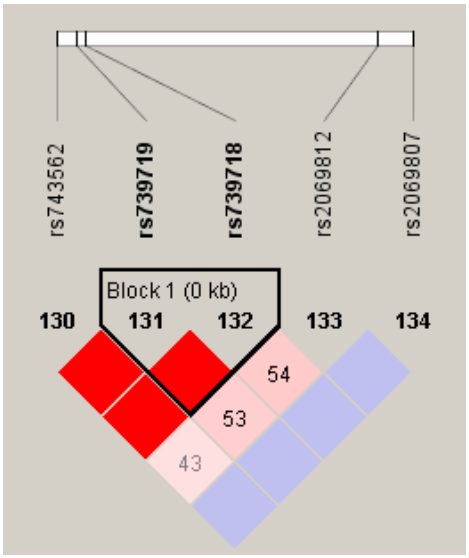
a. Maternal CRHBP Cases



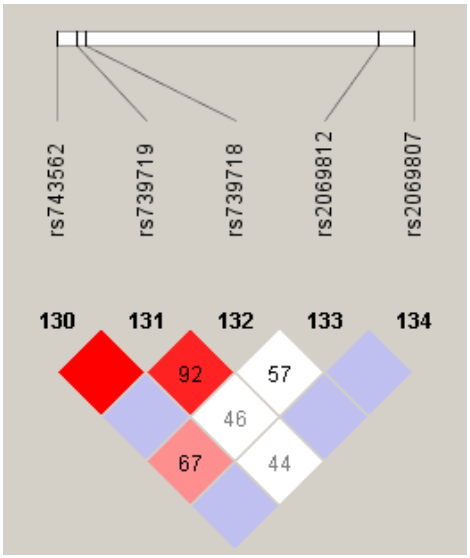
b. Maternal CRHBP Controls



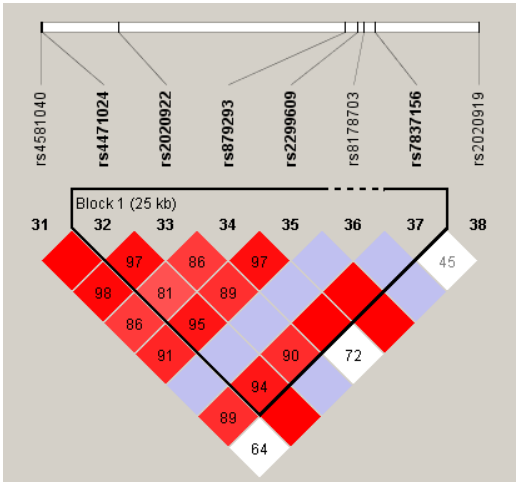
c. Maternal IL-5 Cases



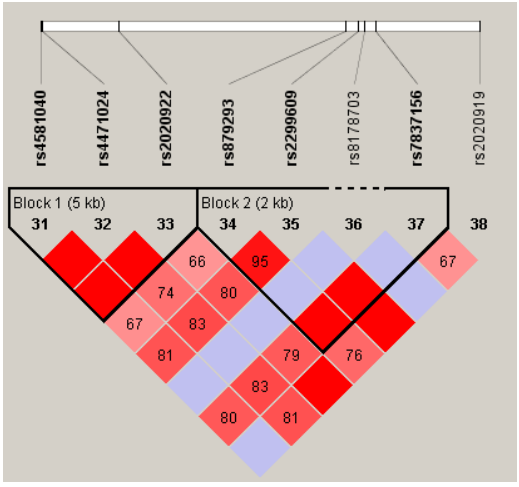
d. Maternal IL-5 Controls



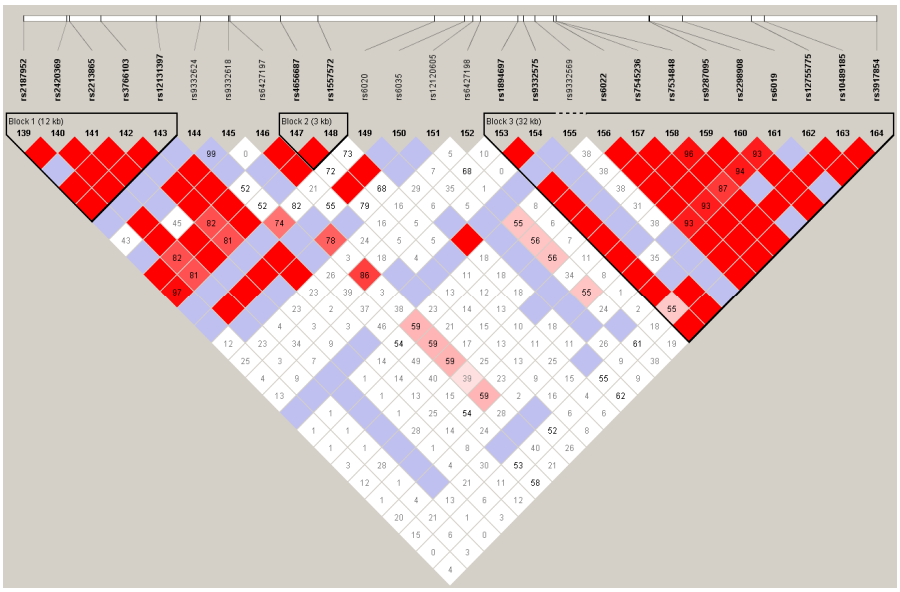
e. Maternal tPA Cases



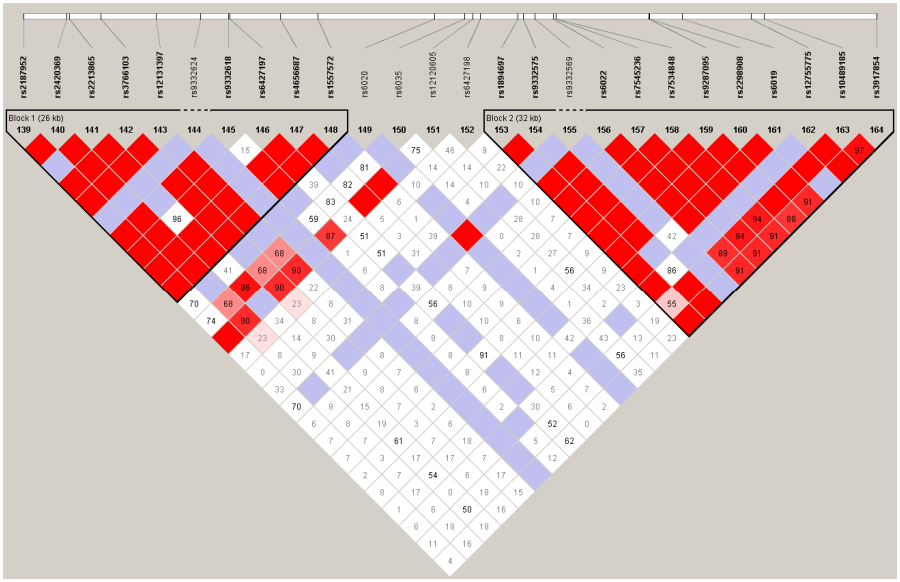
f. Maternal tPA controls



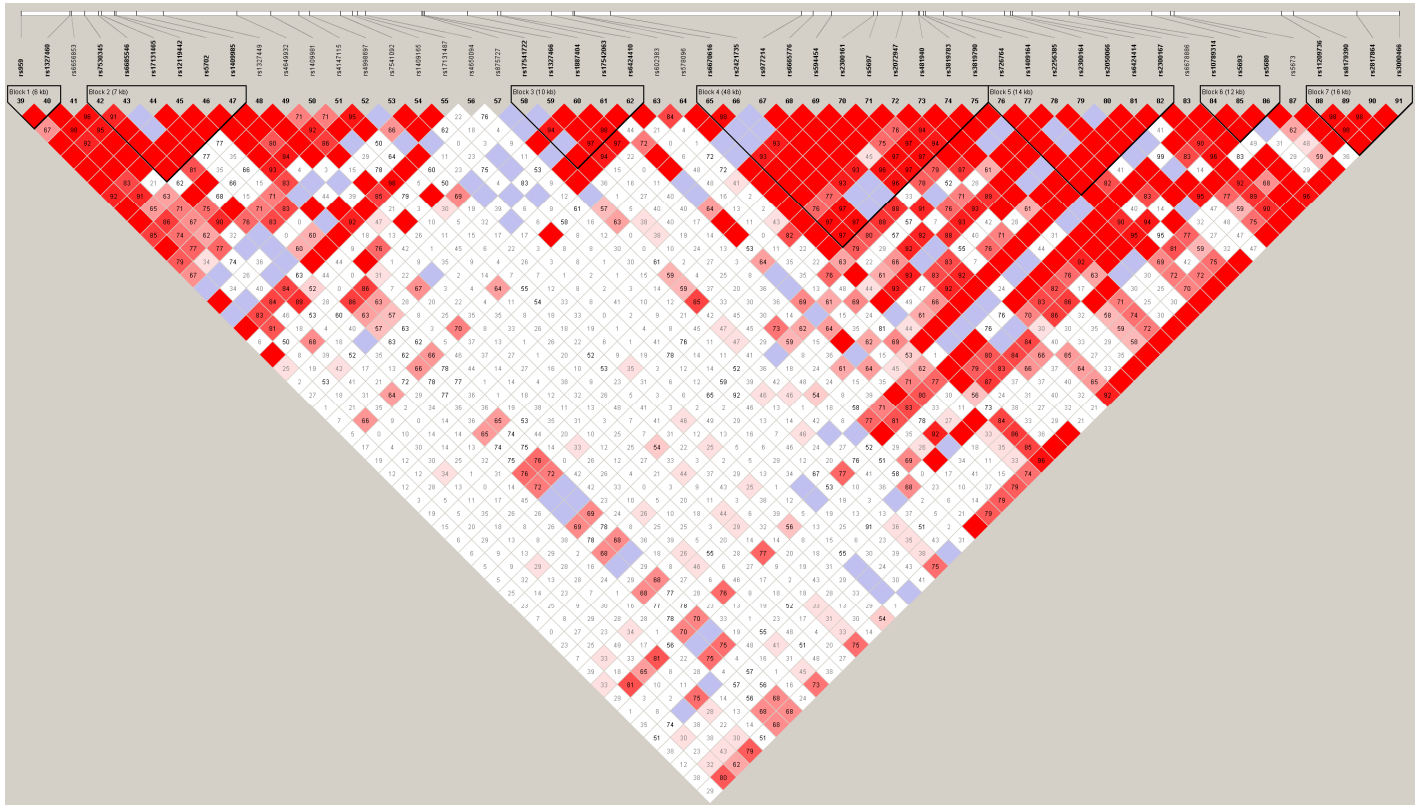
g. Maternal FV Cases



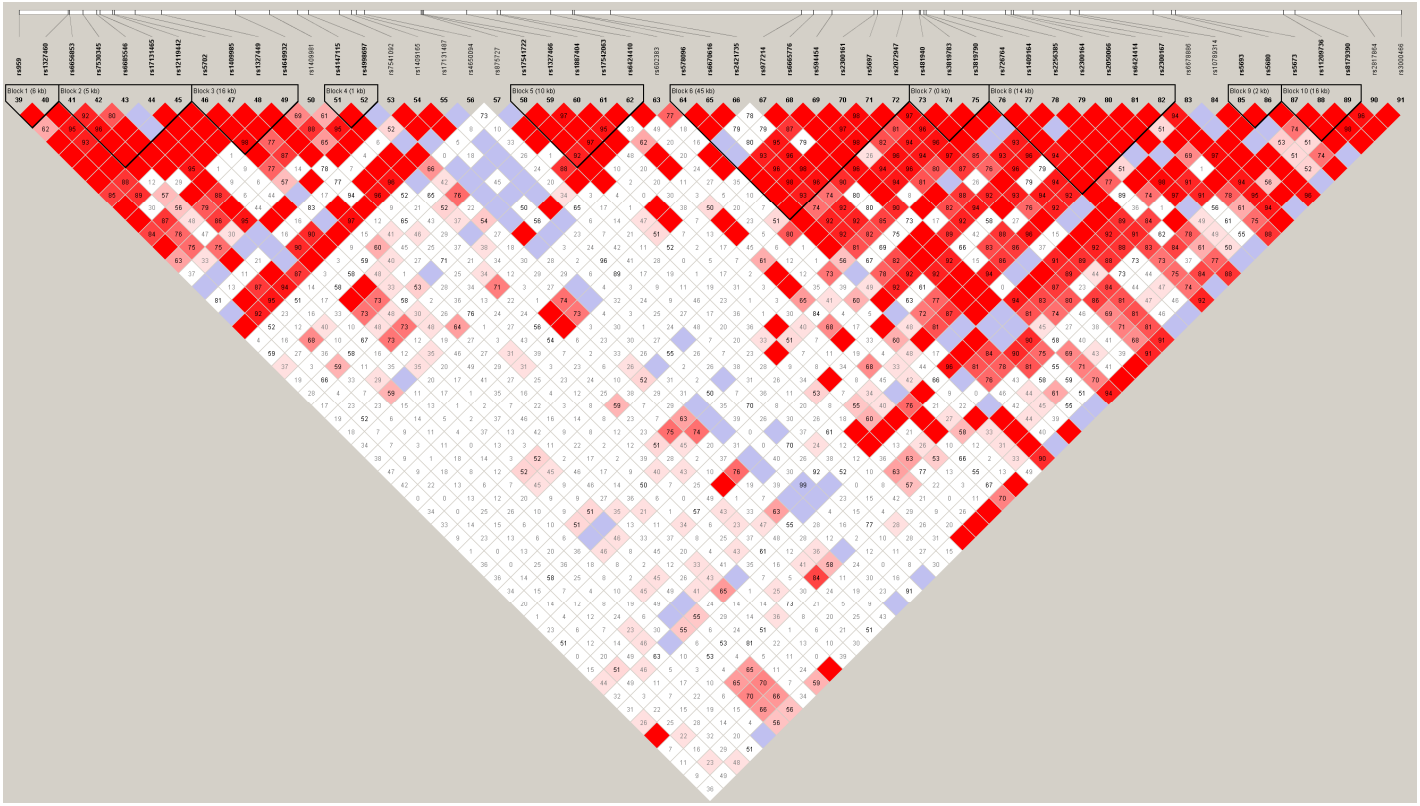
h. Maternal FV Controls



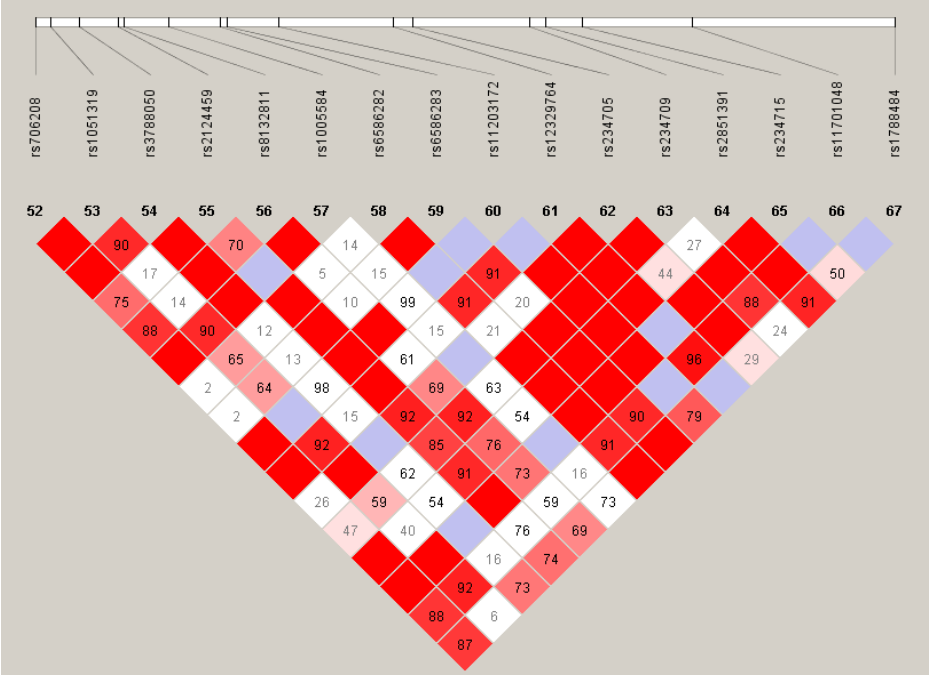
I. Maternal PTGER3 Cases



j. Maternal PTGER3 Controls

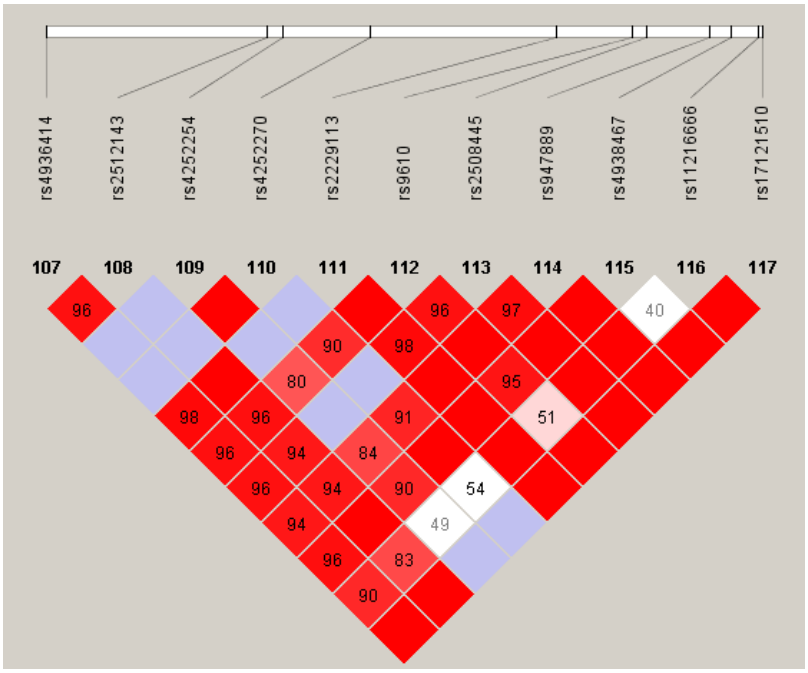


k. Fetal CBS Cases



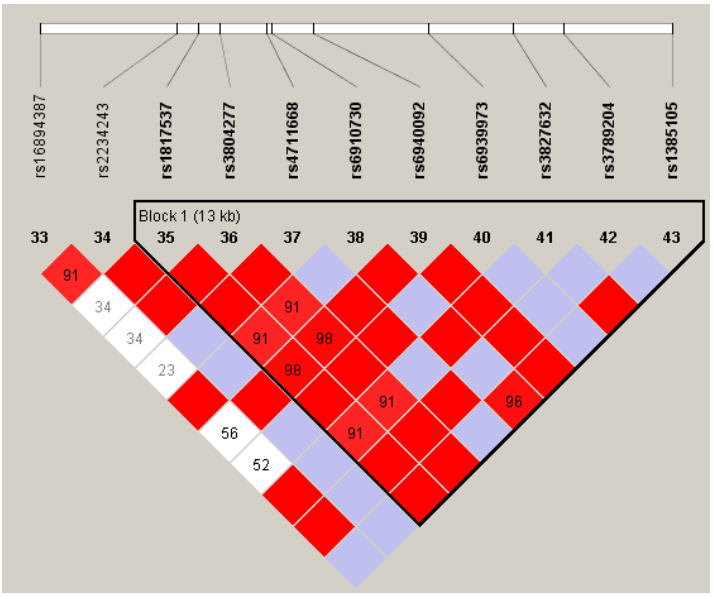
l. Fetal CBS Controls

m. Fetal IL-10RA Cases

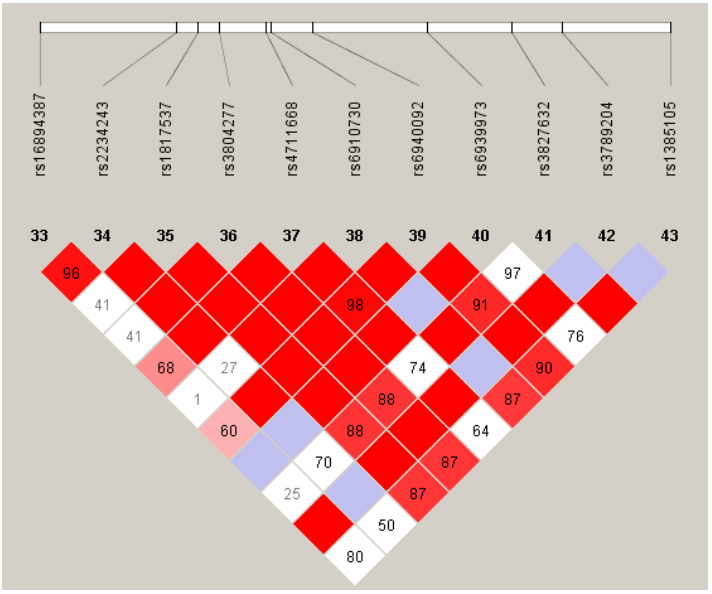


n. Fetal IL-10RA Controls

o. Fetal TREM1 Cases

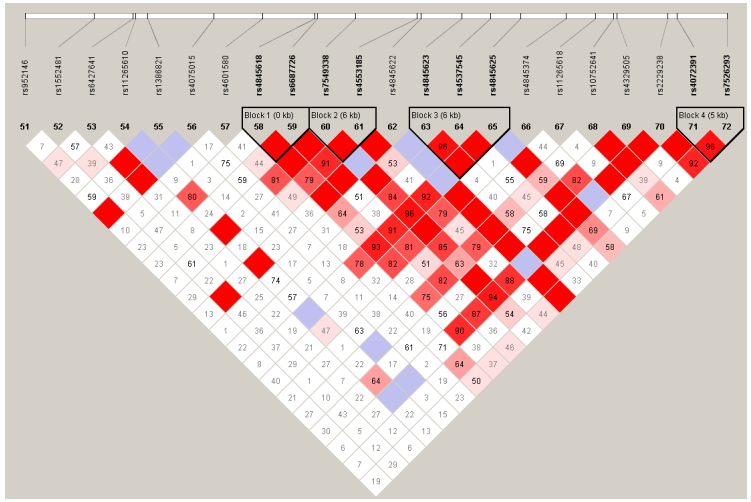


p. Fetal TREM1 Controls

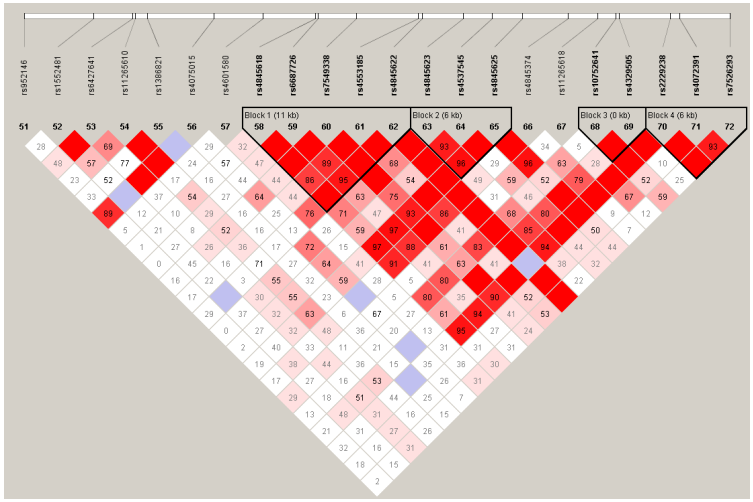


Appendix Figure 3. Haplotype block structure Caucasians.
a-j) Caucasian maternal Haploview LD plots for cases and controls; k-p) Caucasian fetal Haploview LD plots for cases and controls. D' values are labeled within each square.

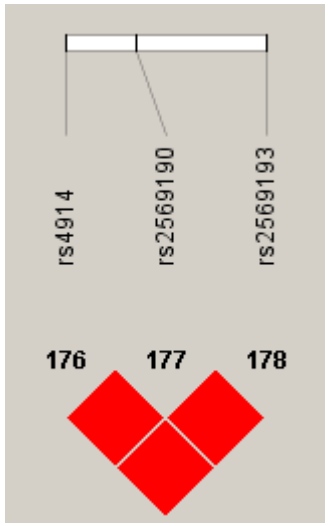
a. Maternal IL-6R Cases



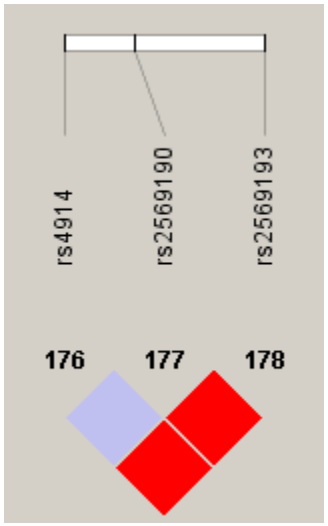
b. Maternal IL-6R Controls



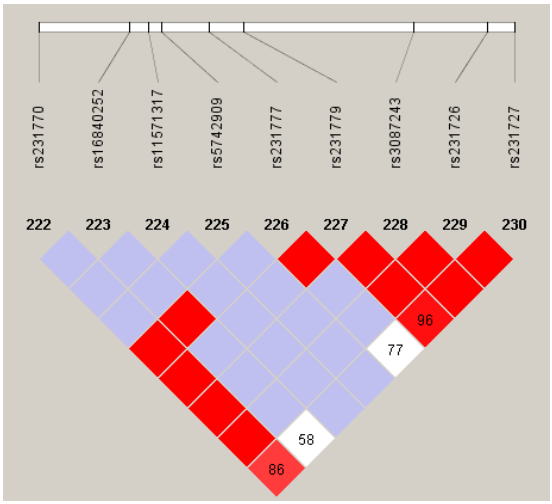
c. Fetal CD14 Cases



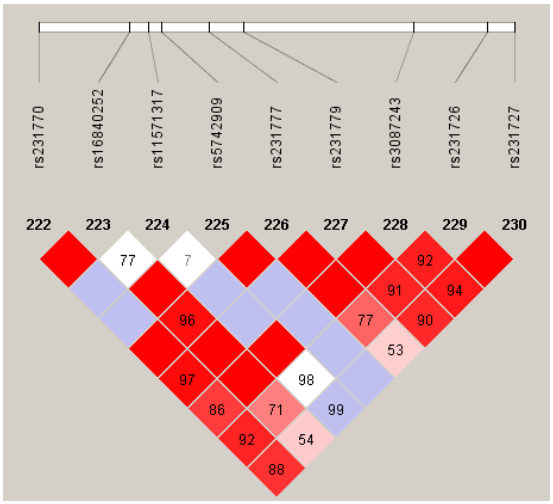
d. Fetal CD14 Controls



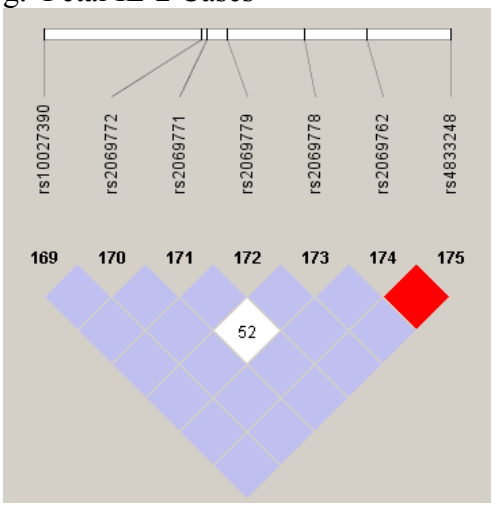
e. Fetal CTLA4 Cases



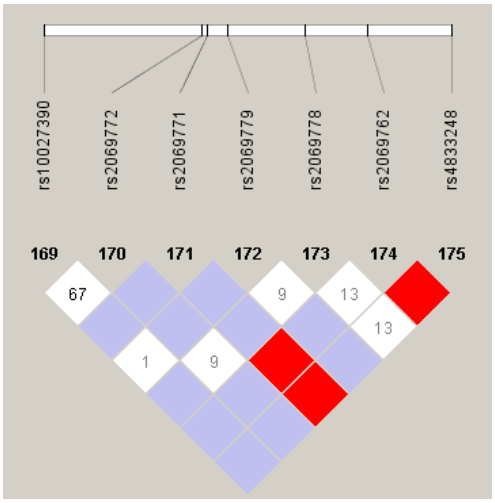
f. Fetal CTLA4 Controls



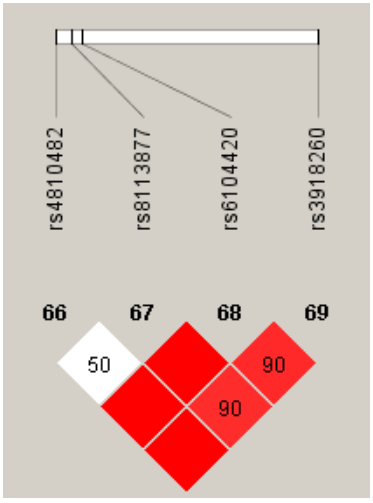
g. Fetal IL-2 Cases



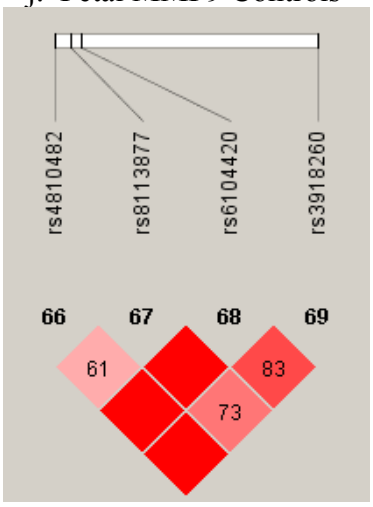
h. Fetal IL-2 Controls



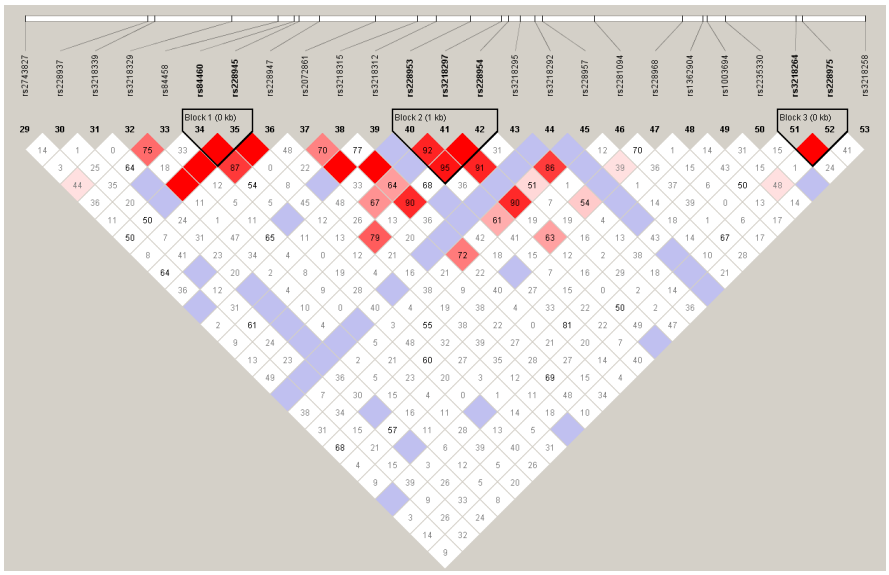
i. Fetal MMP9 Cases



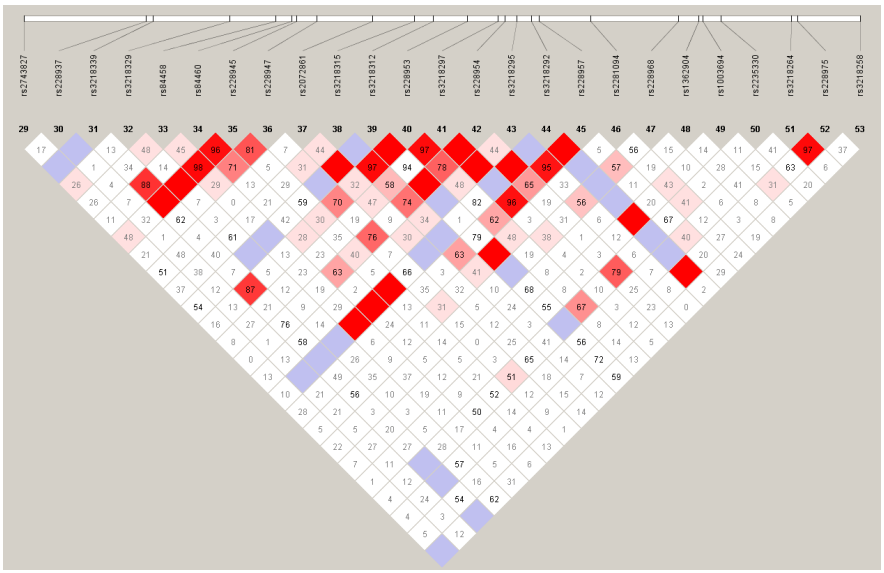
j. Fetal MMP9 Controls



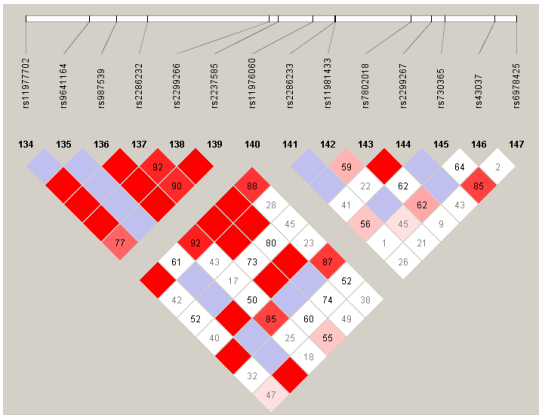
k. Fetal IL-2RB Cases



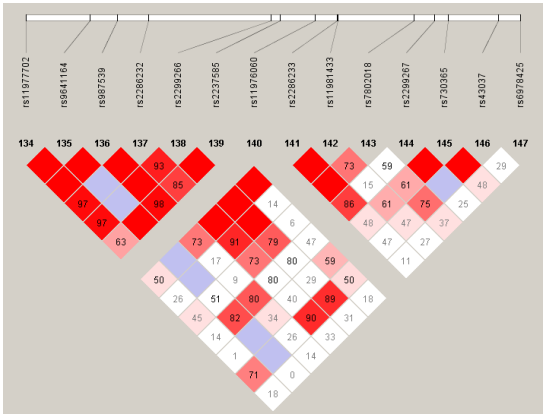
l. Fetal IL-2RB Controls



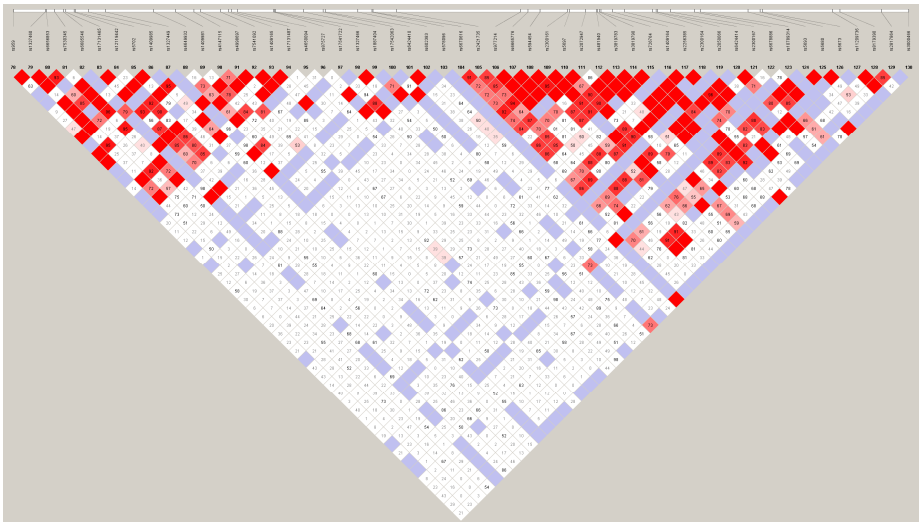
m. Fetal PON2 Cases



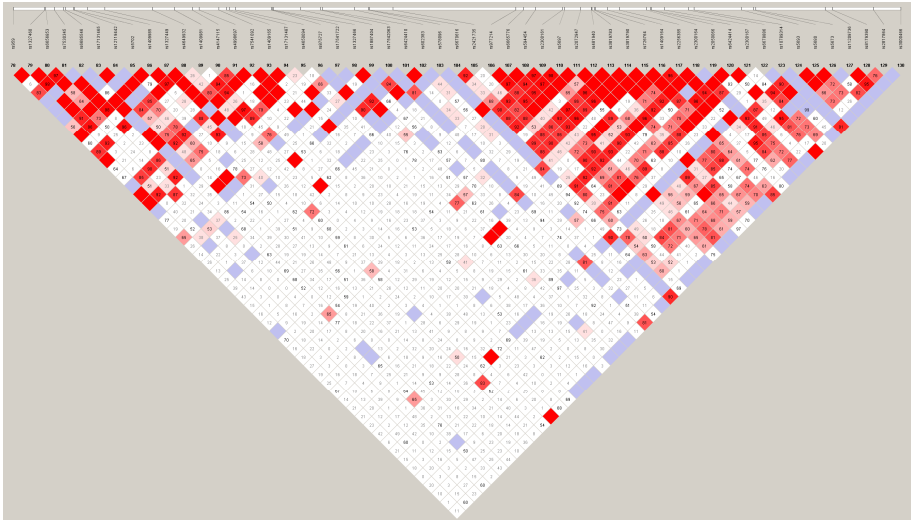
n. Fetal PON2 Controls



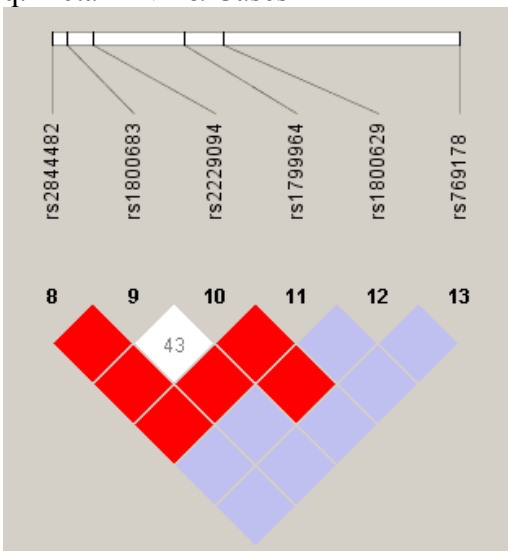
o. Fetal PTGER3 Cases



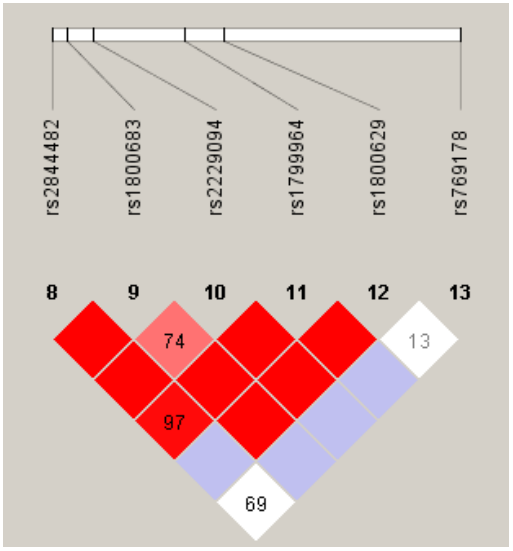
p. Fetal PTGER3 Controls



q. Fetal TNF-α Cases



r. Fetal TNF-α Controls



Appendix Figure 4. Haplotype block structure African Americans.

a-b) African American maternal Haploview LD plots for cases and controls; c-r) African American fetal Haploview LD plots for cases and controls. D' values are labeled within each square.

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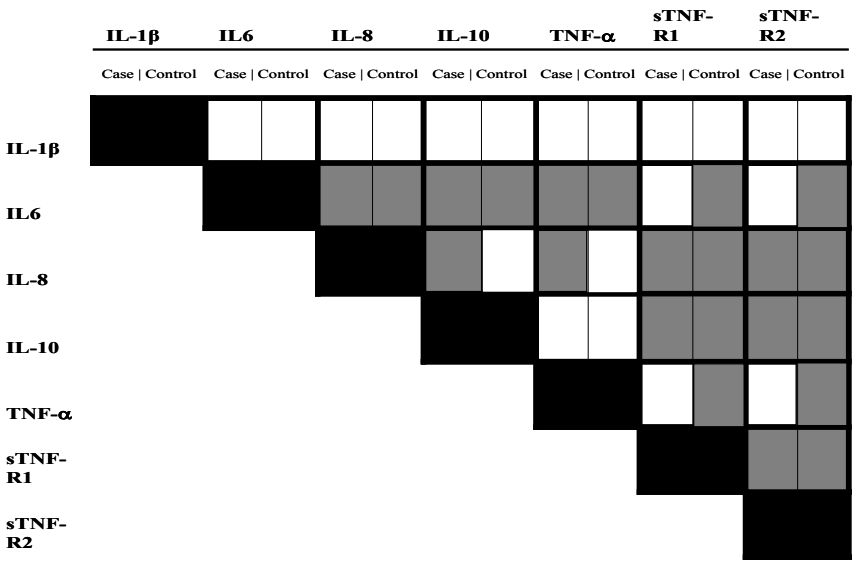
Appendix Table 5. Correlations within cases and within controls

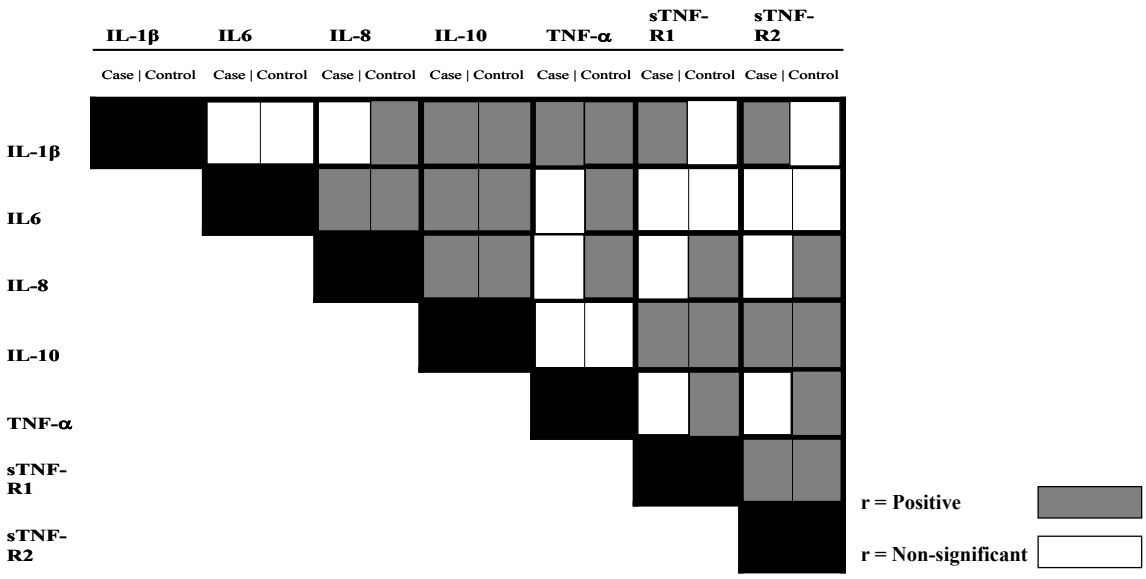
Cytokine 1	Cytokine 2	Cases Caucasian		Controls Caucasian		Cases African-American		Controls African-American	
		r	p	r	P	r	p	r	p
IL-6	IL-1β	0.046	0.661	0.095	0.398	0.070	0.680	0.110	0.313
IL-8	IL-1β	0.153	0.136	0.197	0.055	0.281	0.088	0.394	<0.001
IL-8	IL-6	0.472	<0.001	0.605	<0.001	0.461	0.004	0.624	<0.001
IL-10	IL-1β	0.080	0.440	-0.029	0.789	0.514	0.001	0.349	0.001
IL-10	IL-6	0.196	0.054	0.390	<0.001	0.429	0.006	0.466	<0.001
IL-10	IL-8	0.357	<0.001	0.198	0.070	0.612	<0.001	0.655	<0.001
TNF-α	IL-1β	-0.101	0.330	-0.089	0.424	0.428	0.008	0.163	0.141
TNF-α	IL-6	0.311	0.002	0.366	0.001	-0.199	0.245	0.362	0.001
TNF-α	IL-8	0.420	<0.001	0.114	0.308	0.091	0.593	0.374	0.001
TNF-α	IL-10	0.171	0.092	0.611	<0.001	0.503	0.001	0.537	<0.001
sTNF-R1	IL-1β	-0.156	0.142	-0.032	0.771	0.322	0.052	-0.056	0.607
sTNF-R1	IL-6	0.068	0.517	0.404	<0.001	0.123	0.470	0.049	0.647
sTNF-R1	IL-8	0.233	0.030	0.343	0.001	0.306	0.061	0.249	0.025
sTNF-R1	IL-10	0.456	<0.001	0.637	<0.001	0.669	<0.001	0.239	0.022
sTNF-R1	TNF-α	0.119	0.258	0.260	0.017	0.360	0.031	0.292	0.006
sTNF-R2	IL-1β	-0.055	0.602	-0.101	0.352	0.377	0.020	0.060	0.582
sTNF-R2	IL-6	0.142	0.166	0.347	0.001	0.094	0.575	0.080	0.451
sTNF-R2	IL-8	0.295	0.005	0.345	0.001	0.245	0.133	0.231	0.038
sTNF-R2	IL-10	0.480	<0.001	0.660	<0.001	0.736	<0.001	0.337	0.001
sTNF-R2	TNF-α	0.071	0.487	0.294	0.006	0.432	0.008	0.302	0.005
sTNF-R2	sTNF-R1	0.895	<0.001	0.889	<0.001	0.873	<0.001	0.850	<0.001

*Shaded in grey are the significant correlations

a. Caucasians



b. African Americans



Appendix Figure 5. Positive and negative correlation summary.
Describes the positive and negative correlations observed in the analysis. The x and the y axis indicate the biomarkers/cytokines being correlated. The left portion of each cell represents the case correlations and the right portion o the control correlations. Shaded in grey are the positive correlations; there are no negative correlations. White boxes indicate that the correlation was not statistically significant. a) Describes the correlations in Caucasians; b) describes the correlations in African Americans.

Appendix Table 6. Significant correlations for MIAC

Cytokine 1	Cytokine 2	Caucasian Cases				African American Cases			
		No MIAC		MIAC		No MIAC		MIAC	
		r	p	r	p	r	p	r	p
IL-6	IL-1β	-0.003	0.978	0.212	0.345	-0.048	0.804	0.429	0.337
IL-8	IL-1β	0.083	0.480	0.316	0.162	0.170	0.378	0.690	0.058
IL-8	IL-6	0.449	<0.001	0.425	0.049	0.405	0.029	0.667	0.050
IL-10	IL-1β	0.022	0.855	0.267	0.229	0.350	0.058	0.905	0.002
IL-10	IL-6	0.306	0.009	-0.025	0.907	0.328	0.076	0.700	0.036
IL-10	IL-8	0.339	0.004	0.528	0.012	0.569	0.001	0.709	0.022
TNF-α	IL-1β	-0.201	0.090	0.140	0.533	0.168	0.385	0.179	0.702
TNF-α	IL-6	0.387	0.001	0.146	0.496	-0.430	0.020	0.643	0.119
TNF-α	IL-8	0.482	<0.001	0.365	0.095	-0.101	0.604	0.238	0.570
TNF-α	IL-10	0.139	0.242	0.192	0.369	0.276	0.140	0.548	0.160
sTNF-R1	IL-1β	-0.198	0.103	-0.173	0.466	0.109	0.574	0.357	0.432
sTNF-R1	IL-6	0.071	0.563	0.012	0.958	0.064	0.741	-0.048	0.911
sTNF-R1	IL-8	0.204	0.101	0.298	0.202	0.294	0.121	-0.117	0.765
sTNF-R1	IL-10	0.408	<0.001	0.513	0.015	0.619	<0.001	0.333	0.381
sTNF-R1	TNF	0.069	0.568	0.171	0.446	0.208	0.279	0.214	0.645
sTNF-R2	IL-1β	-0.086	0.470	0.005	0.984	0.105	0.581	0.750	0.052
sTNF-R2	IL-6	0.147	0.219	0.060	0.779	0.020	0.917	0.333	0.420
sTNF-R2	IL-8	0.238	0.049	0.503	0.017	0.167	0.378	0.417	0.265
sTNF-R2	IL-10	0.430	<0.001	0.670	<0.001	0.684	<0.001	0.800	0.010
sTNF-R2	TNF-α	0.058	0.625	0.084	0.697	0.249	0.185	0.179	0.702
sTNF-R2	sTNF-R1	0.881	<0.001	0.899	<0.001	0.870	<0.001	0.633	0.067

Appendix Table 7. Correlation comparisons

Cytokine 1	Cytokine 2	Caucasian v African-American Cases p	Caucasian v African-American Term p	Caucasian Cases v Caucasian Term p	African-American Cases v African-American Term p
IL-6	IL-1β	0.903	0.920	0.750	0.843
IL-8	IL-1β	0.504	0.179	0.769	0.532
IL-8	IL-6	0.941	0.850	0.224	0.254
IL-10	IL-1β	0.013	0.011	0.469	0.305
IL-10	IL-6	0.185	0.549	0.160	0.818
IL-10	IL-8	0.083	<0.001	0.258	0.722
TNF-α	IL-1β	0.005	0.109	0.936	0.153
TNF-α	IL-6	0.010	0.977	0.684	0.005
TNF-α	IL-8	0.077	0.084	0.031	0.144
TNF-α	IL-10	0.054	0.476	<0.001	0.819
sTNF-R1	IL-1β	0.015	0.878	0.416	0.055
sTNF-R1	IL-6	0.786	0.014	0.019	0.714
sTNF-R1	IL-8	0.694	0.513	0.440	0.758
sTNF-R1	IL-10	0.109	0.001	0.086	0.004
sTNF-R1	TNF-α	0.205	0.826	0.336	0.712
sTNF-R2	IL-1β	0.023	0.296	0.756	0.094
sTNF-R2	IL-6	0.804	0.068	0.150	0.947
sTNF-R2	IL-8	0.787	0.433	0.715	0.943
sTNF-R2	IL-10	0.031	0.004	0.071	0.003
sTNF-R2	TNF-α	0.051	0.954	0.126	0.460
sTNF-R2	sTNF-R1	0.608	0.298	0.836	0.653

*Shaded in grey are statistically significant differences between correlations

Appendix Table 8. Correlation comparisons between MIAC and no MIAC

Cytokine 1	Cytokine 2	Caucasian MIAC v No MIAC P	African-American MIAC v No MIAC P
IL-6	IL-1β	0.371	0.229
IL-8	IL-1β	0.324	0.090
IL-8	IL-6	0.904	0.325
IL-10	IL-1β	0.301	0.004
IL-10	IL-6	0.147	0.166
IL-10	IL-8	0.339	0.511
TNF-α	IL-1β	0.156	0.978
TNF-α	IL-6	0.267	0.004
TNF-α	IL-8	0.559	0.389
TNF-α	IL-10	0.817	0.405
sTNF-R1	IL-1β	0.919	0.530
sTNF-R1	IL-6	0.810	0.780
sTNF-R1	IL-8	0.693	0.271
sTNF-R1	IL-10	0.584	0.321
sTNF-R1	TNF-α	0.672	0.987
sTNF-R2	IL-1β	0.708	0.039
sTNF-R2	IL-6	0.711	0.412
sTNF-R2	IL-8	0.204	0.469
sTNF-R2	IL-10	0.137	0.490
sTNF-R2	TNF-α	0.912	0.860
sTNF-R2	sTNF-R1	0.720	0.124

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