MATERNAL AND NEWBORN POLYMORPHISMS IN PHASE I/II METABOLIC GENES CONTRIBUTE TO RISK OF ADVERSE REPRODUCTIVE OUTCOMES

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MATERNAL AND NEWBORN POLYMORPHISMS IN PHASE I/II METABOLIC GENES CONTRIBUTE TO RISK OF ADVERSE REPRODUCTIV OUTCOMES

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Maternal cigarette smoke exposure during pregnancy has been identified as a risk factor for adverse reproductive outcomes, a major public health concern. However, little is known about genetic susceptibility and possible interactions with environmental factors to increase risk of these events. This study was designed to investigate relative contributions of genetic and maternal environmental risk factor interactions to adverse reproductive outcomes. Maternal peripheral and umbilical cord blood samples from 1148 healthy mother/newborn pairs were genotyped for a panel of polymorphisms associated with the metabolic enzymes *CYP1A1*, *CYP2E1*, *GSTM1*, *GSTT1* and *NAT2** for several subgroups; low birthweight (<2500g, n=86), preterm delivery (<37th gestational week, n=93), premature birth (<2500g & <37th gestational week, n=53) and small for gestational age (SGA) at term (≥37th gestational week, n=948) in comparison to the average for gestational age (AGA) group (n=948).

Maternal cigarette smoking during the last trimester was significantly associated with birthweight reduction (μ=101.4g, SE=32, p=0.002). Maternal *GSTT1* null genotype was significantly associated with low birthweight (OR=1.97, 95% CI: 1.24-3.12, p=0.004), preterm

delivery (OR=1.91, 95% CI: 1.22-2.98, p=0.004) and premature birth (OR=2.42, 95% CI: 1.38-4.26, p=0.002). The mean reduction of birthweight observed among the maternal *GSTT1* null genotype group was 89.6g (SE=37, p=0.018) and the mean reduction in gestational age was 0.25 weeks (SE=0.1, p=0.049). In addition, African American women were more likely to have a smaller baby; the mean reduction of birthweight was 230g (SE=34.5, p<0.001) compared with Caucasians. An additive interaction between smoking, African American ethnicity and *GSTT1* null genotype was observed (OR=7.81, 95% CI: 2.49-24.43, p<0.001). The mean birthweight reduction observed in this group was 570.0g (SE=117, p<0.001) and the mean gestational age reduction was 1.10 weeks (SE=0.4, p=0.007). A similar risk was observed for newborn *GSTT1* null genotype in the presence of maternal smoking (426.7g,SE=111, p<0.001) and (1.0 weeks, SE=0.4, p=0.012).

These results demonstrated a clear overrepresentation of maternal and newborn *GSTT1* null genotype among adverse reproductive outcome cases. Furthermore, a gene-gene-environment interaction was observed where the combination of maternal and newborn *GSTT1* null genotype in the presence of maternal cigarette smoke during pregnancy significantly increased risk of adverse reproductive outcomes.

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4-ABP Hb	4-aminobiphenyl-hemoglobin
AGA	Average for gestational age
AFMU	5-acetylamino-6-formylamino-3-methyluracil
AHH	Aromatic hydrocarbon hydroxylase
BD	1,3-butadiene
bp	base pair
CYP	Cytochrome P450
DCM	Dichloromethane
DEB	[2,2']bioxiranyl (aka 1,2:3,4-dipoxy butane)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EO	Epoxide
FAS	Fetal alcohol syndrome
FISH	Fluorescent *in situ* hybridization
GED	General equivalency diploma
GPA	Glycophorin A
GSH	Glutathione
GST	Glutathione S-transferase
GSTM1	Glutathione S-Transferase mu 1
GSTT1	Glutathione S-Transferase theta 1

GW	Gestational week
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
IUGR	Intrauterine growth retardation
MEB	2-vinyloxirane (aka 3,4-epoxy butane)
MRN	Medical record number
MWH	Magee-Womens Hospital
NAT	N-acetyltransferase
*NAT2**	N-acetyl transferase 2
NATP	N-acetyltransferase pseudogene
NC	Non-conjugators
NER	Nucleotide excision repair
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNAL-Gluc	4-[(methylnitrosamino)-1-(3-pyridyl)but-1-yl]β-*O*-D-glucosiduronic acid
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N'-nitrosonornicotine
OB/GYN	Obstetrics and gynecology
8-OHdG	8-hydroxy-2'-deoxyguanosine
OR	Odds ratio
PAH	Polycyclic aromatic hydrocarbon
PEPP	Prenatal Exposure and Preeclampsia Prevention
RFLP-PCR	Restriction fragment length polymorphism-polymerase chain reaction
RNA	Ribonucleic acid
SCGE	Single cell gel electrophoresis

SE	Standard error
SGA	Small for gestational age
SO	2-phenyloxirane (aka styrene 7,8-oxide)
SNP	Single nucleotide polymorphisms
TBE	Tris Borate EDT

1 INTRODUCTION

1.1 Adverse outcomes in pregnancy

At the beginning of the 20th century, approximately 30% of infants did not survive the first year of life [1]. The causes of infant mortality are strongly associated with adverse reproductive outcomes such as infant prematurity, low birthweight, congenital anomalies including heart disease, Potter's syndrome, and trisomy [2]. Improvement of environmental interventions, nutrition, clinical medicine, standards of living conditions and increases in education levels, resulted in a dramatic decline of infant mortality of more than 90% to approximately 7.2 per 1000 live births in the late 1990's compared with that in the beginning of the century [1]. Unlike infant mortality, however, the rate of low birthweight in the U.S. has not declined significantly in the last decade.

1.1.1 Public health perspective of adverse reproductive outcome

From a public health perspective, improvement of early risk detection and prevention strategies will make it possible to reduce adverse outcomes in pregnancy and support a healthier and better quality of life for both mothers and newborns. Identification of factors associated with a poor pregnancy outcome, would help to identify high risk individuals for targeted monitoring and intervention measures [3]. Reduction of adverse reproductive outcomes would not only reduce substantial expenses at labor and delivery and subsequent intensive care but also reduce lifelong suffering for the affected children and their families.

Despite extensive epidemiological research, factors thought to be associated with adverse reproductive outcomes have not yet been fully characterized. A few known risk factors include maternal health conditions such as diabetes, heart disease and erythroblastosis. However, other factors including lifestyle and behavior factors such as maternal age, nutrition, cigarette smoking and alcohol intake are more controversial [4]. With advancements in medical technology, adverse outcomes caused by maternal health conditions have declined. However, further investigations are necessary in order to better understand the contribution of genetic, environmental, maternal lifestyle and behavioral factors to adverse reproductive outcomes.

1.2 Epidemiology of birthweight and gestational age in the U.S.

In the U.S., the frequency distribution of birthweight is normally distributed with an extended lower tail accounting for approximately 2 to 5% of births [5]. The birthweight distribution for term births (≥37 weeks of gestation) is closely associated with gestational age but less correlated with preterm births (<37 weeks of gestation). The majority of births occuring in the lower tail of the birthweight distribution are preterm births but not exclusively [6]. Wilcox and et al. [5] suggested that the birthweight distribution in preterm and term births are two independent events. For example, an exposure that affects fetal growth causing birthweight reduction does not necessarily affect the risk of preterm delivery.

Birthweight and gestational week are powerful predictors of infant survival and other health conditions in infancy and also in childhood [1, 2, 4, 5, 7]. Approximately 75% of infants who die within their first year after birth, are low birthweight babies and more than half of these

are preterm deliveries [4]. At the population level, the mean birthweight is associated with infant mortality. Groups with lower mean birthweight often have higher infant mortality. In the U.S., interventions to increase birthweight are recommended as a strategy to improve the infant mortality rate [5].

In the year 2000, approximately 7.6% of all infants were born low birthweight, defined as less than 2,500 grams, and the percentage of infants delivered at very low birthweight, defined as less than 1,500 grams, was approximately 1.43% in both singleton and multiple births. Approximately 6.0% of infants were singleton births with low birthweight in 2000 in the U.S. [8].

1.2.1 Intrauterine growth retardation (IUGR) and low birthweight

In general, low birthweight infants are typically 20 or greater times more likely to die than infants of normal birthweight within the first year of life [5]. Mortality ranges more than 100-fold across the spectrum of birthweights and this phenomenon has been observed in all populations and occurs with stillbirth and with neonatal or infant mortality [8, 9]. In addition, it is strongly associated with adverse health outcomes later in life such as asthma [10], low IQ [11, 12] and hypertension [13]. Moreover, extensive epidemiological studies have demonstrated an association between birthweight and adult diseases such as cardiovascular diseases [14], diabetes [15], cancers [16, 17], and impairments of hearing or vision [18].

Reduction of birthweight is commonly caused by the restriction of fetal growth. From a clinical standpoint, infants born weighing less than the tenth percentile for gestational age are

generally classified as growth restricted. The cutoff values are determined by population based standards. However, this classification may not be sensitive and/or specific enough to correctly identify growth retarded infants [19].

The etiologies of fetal growth retardation are complex and still not completely understood. Most growth retardation occurs after the 28th week of gestation, with increasing frequency to term [20]. However, it may occur at any stage of pregnancy. Heins et al. [4] suggested that genetic defects and injury to the developing embryo in early pregnancy might cause infant growth retardation later. Lin [20] described that if growth retardation occurs during the first 16 weeks of embryonic and fetal life, the primary effect is a reduction of cell number and these infants are usually small in size with a reduction of all external measurements including weight, length, and head circumference. This type of growth retardation is frequently associated with congenital malformations, genetic abnormalities and other teratogenic effects with a higher mortality rate [20]. Perinatal mortality for fetuses without chromosome anomalies was approximately 27% while roughly 64% was observed for early onset SGA fetuses with anomalies [21]. Chromosome abnormalities such as Down's syndrome, trisomy 13, and trisomy 18 are more commonly seen among these growth retarded infants [4, 22]. Chromosome loss or additions of extra segments affecting a large number of genes located on the chromosome causes significant disability, including fetal growth retardation. Roughly 7% of infants with growth retardation initiated at any stages of pregnancy had chromosomal abnormalities [21]. Moreover, infants with growth retardation started in early pregnancy have a much higher proportion (19%) of having chromosome abnormalities [22, 23].

From 32 weeks of gestation to term, fetal cell size rapidly increases, along with the rate of glycogen and fat deposition. Growth retardation occurring during this period results in a disproportionately larger head, smaller abdominal organs and a lack of subcutaneous fat [20]. Maternal health conditions such as hypertension, preeclampsia, placental infarcts and multiple pregnancy are strongly associated with this type of fetal growth retardation [20]. The third type of fetal growth retardation that Lin [20] described was the combination of those two types and Lin speculated that the timing of the interaction of risk factors including maternal smoking and alcohol intake, and the stage of gestation plays an important role in the development and progression of fetal growth retardation.

1.2.2 Preterm delivery

Preterm labor and delivery occurs in 6-10% of pregnancies and is the major factor contributing to perinatal morbidity and mortality [24, 25]. Almost one-fifth of all very preterm infants (less than 32 weeks) do not survive the first year of life, compared with about 1% of infants born preterm (32 to 36 weeks) and 0.3% of infants born at term (37 to 41 weeks) [8]. Preterm newborns who do survive are more likely to be neurologically impaired [26]. There are four major causes of preterm delivery: preterm labor, preterm/premature rupture of the membranes, maternal medical or obstetric complications; and fetal distress or demise. The proportion of each event depends on the population characteristics [25]. A large number of risk factors which are summarized below may modulate the proportions of these events.

1.3 Risk factors

Risk factor analyses and identification are a powerful tool to identify high-risk individuals for adverse reproductive outcomes and to reduce these risks for better outcomes. Moreover, characterization of risk factors could be helpful for better understanding the etiologies of these adverse events. Galbraith et al. [27] reported that two thirds of growth retarded infants are derived from the population with known risk factors, and one third come from the population with unknown risk factors.

The risk factors can be roughly categorized into fetal, placental and maternal factors for adverse reproductive outcomes. Fetal factors include genetic conditions and congenital anomalies. Placental factors tend to involve abnormally implanted or formed placentas. Maternal factors include infection, nutrition, uterine perfusion of the placenta, drug use, various medical conditions and certain demographic variables [19].

Maternal medical and obstetric factors tend to have a stronger association with adverse reproductive events. However, maternal demographic and behavioral factors such as maternal age, socioeconomic status, cigarette smoking exposure and alcohol intake during pregnancy are more controversial. Extensive epidemiological studies have been conducted to determine if these maternal lifestyle factors are significantly associated with adverse outcomes [25, 28-31]. Table 1 summarizes a partial list of maternal risks associated with preterm delivery and low birthweight.

Table 1. Risk factors associated with preterm delivery and low birthweight [25, 28-30]

Risk categories	Known risk factors
Demographic	Age
	Race
	Socioeconomic status
	Marital status
Behavioral	Smoking
	Other substance abuse
	Poor nutrition
	Excessive physical activity
Health Care	Absent or inadequate prenatal care
Medical Risks Predating Pregnancy	Poor obstetric history
	Uterine or cervical malformations\myomas/DES exposure
	Selected medical conditions
Current Pregnancy Complications	Multiple gestations
	Abnormalities in amniotic fluid volume
	Vaginal bleeding
	Fetal abnormalities
	Serious infection
	Abdominal surgery

1.3.1 Maternal age

Due to women's career priorities, advanced education, control over fertility, financial concerns, late and second marriages and infertility, increasing numbers of older women are bearing children. The number of pregnancies among the age group 35 to 40 years in the U.S. is projected to increase 37% [32]. Since maternal age is a well-known risk factor of number of adverse reproductive outcomes such as fetus with chromosomal abnormalities [32, 33], incidence of those adverse events is expected to increase. Moreover, maternal age has been found to be a risk factor for fetal growth retardation resulting in low birthweight infants [3, 8, 29, 34].

Not only older women but also younger women are at a high risk for adverse reproductive outcomes. Teenage mothers have an increased risk of low birthweight, preterm delivery and a higher rate of infant mortality [35-39]. For early teenage pregnancy, both maternal biological factors including immaturity of the female reproductive system and inadequate prenatal weight gain [37], together with sociocultural factors [36, 39], and lifestyle choices made by adolescents, combine to influence the risk of delivering a low birthweight infant [35].

There is therefore, a slight U-shaped association of low birthweight status by maternal age. Women under 20 years and over 35 years are at increased risk for a poor pregnancy outcome, compared with women between 25 and 29 years of age [3].

The data summarized in Table 2 shows that low birthweight rates tend to be highest among the youngest (< 15 years old) and the oldest (≥ 40 years old) age groups with the percentage of low birthweight of 14.1% and 18.2% respectively. However, much of the low birthweight risk for the latter age group is attributable to the greater numbers of multiple births [8].

Table 2. Overall percentage of low birthweight among live births by maternal age in year 2000 in U.S. [8]

Maternal age	Proportion of low birthweight infant (%)
<15 years old	14.1
15-19 years old	9.5
20-24 years old	7.6
25-29 years old	6.9
30-34 years old	6.9
35-39 years old	8.3
40-44 years old	10.0
≥45 years old	18.2

1.3.2 Maternal ethnicity and socioeconomic status

There are significant differences in low birthweight, preterm delivery and mortality rates among different racial groups. African American infants are more than twice as likely to die as Caucasian infants [1, 40-47]. The median birthweight for all live births for the year 2000 in the U.S. [8] was 3,350 grams (3,390 grams in Caucasians and 3,180 grams in African Americans). Numerous research studies have been undertaken to elucidate the reasons for this disparity.

There is a clear association between maternal ethnicity and preterm delivery. The percentage of preterm African American neonates born prior to 32 weeks' gestation is higher than that for Caucasian preterm neonates (23% vs. 17%). In addition, African American women are more likely to deliver at a younger age, are less educated, and have a lower socioeconomic status. Thus, other risk factors such as maternal age and socioeconomic status significantly interact with ethnicity [25].

Much of the disparity between the races in the frequency of preterm delivery and low birthweight has been attributed to differences in socioeconomic status. Lower socioeconomic status may be associated with poorer nutrition, increased exposure to infectious agents, adverse environmental factors, and increased stress during pregnancy [47]. Moreover, some maternal risk behaviors including cigarette smoking, alcohol consumption and avoiding prenatal care, may be related to socioeconomic status. Among the U.S. population, a higher percentage of African American women have lower socioeconomic status than Caucasians. However, James [45] has reported that despite similar socioeconomic profiles including economic disadvantages and reduced access to prenatal care, infant mortality among Mexican Americans is less than half that observed in African Americans. He suggested that there are unknown risk factors unique to the African American community and lifestyles or some other protective social and psychological content of Mexican culture [45].

Lower socioeconomic status could also affect maternal health status at the time of conception. Rawlings et al. [43] has reported that African American women tend to require a longer time between pregnancies than Caucasians to achieve an optimal outcome. This difference could reflect lower physiologic reserves [47]. However, African American women are approximately 1.6 times as likely as Caucasian to have shorter interval between deliveries [43, 47].

Lieberman [47] also suggested that the adverse pregnancy outcomes affected by maternal health status may reflect not only her recent behavior but also her lifetime health status and even for over a number of generations. Wang et al. [42] has reported that there is a significantly

higher risk of having low birthweight infant if the mother was also a low birthweight infant herself in both African Americans and Caucasians in the U. S.

Differences in adverse pregnancy outcomes continue to exist even after the adjustment of the mother's socioeconomic status [48]. The excess risk for low birthweight among African Americans as compared with Caucasian women has been noted to be greater among low risk mothers, classified as the absence of high level of cigarette smoking and other harmful exposures, than among high-risk mothers [46]. Wilcox [49] suggested that there might be involvement of genetic factors modulating adverse reproductive outcomes based on ethnic groups. Our understanding of racial differences in the occurrence of low birthweight and the underlying biological mechanisms of adverse events is very limited.

While various factors have been found to be associated with increased risk for having a low birthweight infant, including maternal anthropometrics, health and age, prenatal care, and socioeconomic status, none have been found to entirely and adequately explain the continued birthweight differential observed between African Americans and Caucasians.

1.3.3 Maternal alcohol consumption during the pregnancy

In 1973, Jones et al. described a constellation of abnormalities termed the fetal alcohol syndrome (FAS), which provided a clear recognition and acceptance of developmental toxicity caused by alcohol [50-52]. Since then, extensive clinical, epidemiological and experimental studies of alcohol effects on fetal adverse outcomes have been demonstrated. FAS comprises craniofacial dysmorphism, include prenatal and postnatal growth retardation, retarded

psychomotor and intellectual development, and other nonspecific major and minor abnormalities [51, 52]. FAS has been described in all races and countries. The estimated worldwide incidence of FAS is 1.9 per 1000 live births. FAS rates in the U.S. vary from approximately 2.5% to 4%. Moreover, in utero exposure to low levels of ethanol has been associated with a wide range of effects, including isolated components of FAS and milder forms of neurological and behavioral disorders [51]. In addition, several studies have been reported which show that alcohol consumption also affects on birthweight. Little [53] prospectively studied 800 women to evaluate the effects of drinking on birthweight. After adjusting for other known risk factors including smoking, gestational age, maternal height, age, parity and sex of the fetus, a dose-related association was observed, such that each ounce of absolute ethanol consumed per day during late pregnancy reduced 160 g of birthweight. However, the mechanism of alcohol's effects on preterm delivery and fetal growth retardation is not fully understood.

1.3.4 Caffeine

Caffeine is widely used in multiple different beverages, including coffee, tea, many carbonated soft drinks and also in chocolate. Caffeine consumption in the U. S. is estimated to be 4.5 kg/person/year [54]. The per-capita consumption of caffeine from all sources is estimated to be about 3–7 mg/kg per day, or ~200 mg/day [55]. Consumption of caffeinated beverages during pregnancy is quite common and is estimated to be approximately 144 mg/day, or 2.4 mg/kg for a 60 kg human [56]. In newborns, the plasma half-life for caffeine is 4 days [57]. Among adults, the half-life averages 2-6 h in healthy nonsmokers, although its metabolism is prolonged in pregnant women to 10-20 h [58].

Extensive epidemiological studies have reported some evidence that caffeine consumption during pregnancy causes fetal growth retardation and leads to low birthweight in offspring [59-62]. Total caffeine consumption of ≥300 mg daily during pregnancy has been associated with reduced birthweight, increased risk of low birthweight infants, and/or IUGR in case control [61], prospective [63-65], and retrospective studies [66-68]. Peacock et al. [69] demonstrated a 6% reduction in birthweight attributable to a caffeine intake of ≥1400 mg per week after adjustment for smoking and alcohol consumption. Martin et al. reported a dose response of caffeine intake to increased risk of low birthweight with heavy caffeine use was associated with a mean of 105 g reduction in birthweight [63]. The adjusted relative risk of having a low birthweight infant was 1.4 for ≤150 mg of caffeine per day, 2.3 for 151-300 mg, and 4.6 for >300 mg daily. Corresponding decreases in mean birthweight were 6 g, 31 g, and 105 g, respectively. In addition, Fenster et al. [70] has demonstrated that caffeine intake in early pregnancy also causes fetal growth retardation. Women who reduced caffeine intake to ≤300 mg/day within 6 weeks of their last menstrual period reduced the risk of having low birthweight newborns compared with women who did not reduce their intake early in their pregnancy.

Caffeine metabolism occurs in the liver via microsomal cytochrome P450 (CYP) monoxygenases and the soluble enzyme xanthine oxidase. Several studies have indicated that newborns are extremely deficient in metabolizing caffeine and postnatal age, gestational age and birthweight of premature infants are significant covariates in the maturation of caffeine metabolism [71-73]. The slow metabolism of caffeine in premature infants is probably related to the low activity of the hepatic enzymes, and the increase of caffeine metabolism is most likely

related to maturation of the hepatic enzymes. Thus, even low quantities of caffeine could lead to toxic effects in newborns [71].

Despite extensive research of caffeine and fetal growth retardation and prematurity, the etiologies still remain unclear. Two biological mechanisms for these adverse reproductive outcomes have been hypothesized. Given the chemical similarity of caffeine to the purine components of nucleic acids, caffeine may interfere as a genotoxic agent. Several studies have reported that caffeine is a well-established inhibitor of DNA synthesis and repair in lower organisms and simple cell systems. However, these phenomena have not been observed in mammalian models. Secondly, caffeine may cause vasoconstriction of placental intervillous blood flow, which in turn, may contribute to the potential risk of hypoxia and fetal growth retardation [56, 59, 74].

1.3.5 Cigarette smoke exposure

Cigarette smoking is one of the most strongly associated preventable risk factors for many adverse reproductive outcomes [75, 76]. Cigarette smoke is known to contain thousands of mutagenic, carcinogenic and teratogenic compounds that may exert toxic effects affecting on reproductive outcomes. The potential effects of cigarette smoke exposure on reproductive outcomes are a major scientific and public health concern. For example, nicotine is known to be vasoactive and is thought to reduce placental and fetal circulation [76]. Carbon monoxide is known to deplete both maternal and fetal oxygen supplies [77, 78]. Moreover, lead is a known neurotoxin and some polycyclic aromatic hydrocarbons are mutagenic [79, 80].

Both active and passive cigarette smoke exposure during pregnancy are known as the strongest preventable causative factors of fetal growth inhibition resulting in preterm delivery and low birthweight [28-30, 81-83]. In 1957, Simpson et al. first reported an association between smoking and preterm labor/delivery [84]. Several studies have demonstrated that the risk of delivering at <37 weeks of gestation was increased an estimated 30% among women who smoked during pregnancy and approximately 13 to 20% of all preterm births could be attributed to maternal smoking [25, 76]. Moreover, according to the National Vital Statistics Report for the year 2000 [8], the association of smoking and low birthweight has been shown in birth certificate data as well as in other studies. The incidence of low birthweight among infants born to smokers was two-thirds higher than that for nonsmokers, 11.9% compared with 7.2% respectively [8]. Moreover, 11.2% of infants born to light smokers (1 to 5 cigarettes per day) are low birthweight, which is approximately 56% higher than that for nonsmokers [8].

The etiologies of IUGR caused by maternal cigarette smoking are still unclear. Some of the mechanisms by which cigarette smoking may affect birthweight are reduction of expanding plasma volume which increases maternal plasma carbon monoxide concentration and as a consequence increases fetal blood carbon monoxide, increasing maternal fetal blood viscosity [85].

1.4 Cigarette consumption in the U.S.

Cigarette smoking is known as the most widely recognized preventable human health hazard for coronary heart disease, stroke, cancer, chronic obstructive lung disease and other circulatory diseases [86]. Cigarette smoking was first introduced to the U.S. in the mid 19th

century and became increasingly popular among adult men, followed by adult women in the first half of the 20th century. The average annual consumption of cigarettes reached its highest levels of 4345 per adult man in the mid 1960's and has gradually decreased after the first Surgeon General's report describing smoking as a cancer causing agent [87]. The average annual consumption among adult men in the U.S. has dropped to 2261 in 1998 [88]. In 1999, an estimated 46.5 million adults (23.5%) in the US. were current smokers and the prevalence of smoking was highest among the younger generation [88-90]. The cigarette smoking prevalence rate varies according to ethnicity, socioeconomic status and educational levels. Among different races, Native American/Alaska natives have the highest smoking prevalence in both men and women followed by African American and Caucasians, and the Asian/Pacific Islanders with the lowest rates [88-90]. In addition to this, the smoking prevalence was highest among the group with less than 12 years of education and lower social economic status in all races in the US. [88].

1.4.1 Cigarette consumption among reproductive-age-women

Cigarette smoking among women began to increase in the 1920's following public acceptance of female smokers. Cigarette consumption among females reached a peak in 1965 and then gradually decreased following the decline of male smoking prevalence [87]. In the 1950s, the ratio of smokers between men and women was 5:1, but in the 1990s, the ratio was close to 1:1. Since 1990, smoking prevalence among both men and women has remained constant (approximately 28.0% for men and 22.5% for women) [88, 89, 91, 92]. In 1999, approximately 21.5% of reproductive-age-women were current smokers and more than half continued smoking during pregnancy [89, 91]. Approximately 30% of pregnant women who

smoke during the 1st trimester quit smoking for the duration of the pregnancy. Unfortunately, up to 60% of these women will return to smoking within the first 6 months postpartum and 80% to 90% will have experienced a relapse by 12 months postpartum. The women who quit smoking at the 3rd trimester are more likely to start smoking after the delivery compared to the women who quit smoking at an earlier time point during pregnancy [93]. Smoking prevalence among pregnant women is further stratified by level of education, marital status and maternal age. Young, unmarried women who have lower education levels are more likely to continue smoking during pregnancy [93]. Moreover, among women aged 17 to 18 years, the smoking prevalence has increased from 1968 to 1979 and in the 1990s, female adolescents were smoking at a rate that surpassed those of male adolescents [92, 94]. Compared with older women, young pregnant women were more likely to be active cigarette smokers and least likely to quit when they became pregnant [92]. Therefore, smoking during the reproductive age of women continues to be a major public health concern. Extensive epidemiological studies have been done in the area of maternal and newborn health defects caused by smoking due to public health concerns regarding the effect of smoking on women's health as well as the effect on fetuses when women smoke during pregnancy and/or are exposed to environmental tobacco smoke.

1.5 Smoking effects and reproductive outcomes

Due to environmental interventions, improvements in nutrition, advances in clinical medicine, improvements in access to health care, improvements in surveillance and monitoring of diseases, increases in education levels, and improvements in standard of living maternal and infant mortalities had dramatically dropped in the last century [1, 95]. Cigarette smoke, which is known as a major preventable risk factor of causing number of different health problems, is also

associated with many adverse reproductive health effects including infertility [96], ectopic pregnancy [97, 98], spontaneous abortion [99], placenta previa [75] and abruptio placentae [91].

1.5.1 Smoking effects on newborns

The risks of maternal lifestyle including alcohol consumption and cigarette smoking during pregnancy on fetal health and development have been extensively studied. Maternal smoking is known to reduce fetal oxygenation through increased blood levels of carboxyhemoglobin and through impairment of oxygen unloading [100, 101]. Nicotine, which is one of the major chemical substances in tobacco products, and is metabolized by phase I metabolic enzymes, also causes vasoconstriction, which in turn causes cyclin synthesis, increased vascular resistance and decreased fetal blood flow [85]. Since the placenta itself requires approximately 50% of oxygen and glucose extracted from uterine circulation under normal conditions, reducing these substrates' availabilities causes reduction of oxygen and glucose and increases the metabolism of amino acids, establishing a complex balance of nutrient utilization between itself and the fetus [85]. Therefore, maternal smoking could cause damage to the developing fetal organs, making them vulnerable to disease, or it may interfere with the immune system, predisposing the child to infections or other diseases [102].

Several adverse prenatal outcomes caused by maternal cigarette smoking during pregnancy which have been investigated include intrauterine growth retardation (IUGR) [85], prenatal mortality [75], preterm delivery [103], sudden infant death syndrome [104] congenital malformations [105], central nervous system defects [106], cardiac defects [107], gastroschisis [108], limb reduction defects, oral clefts [109], learning behavioral and attention disorders and

mental retardation [12, 51, 110]. In addition, extensive epidemiological studies have been under taken to investigate potential associations between maternal cigarette smoking and childhood cancer especially leukemia [17, 111-113] and cancer in early adulthood [114-116].

1.5.1.1 Cigarette smoking and fetal growth retardation and preterm delivery

Smoking during pregnancy is one of the strongest preventable risk factors of fetal growth retardation that is associated with preterm delivery and low birthweight infant. The reported reduction of birthweight caused by maternal cigarette smoking is an average of 200 g, and there is a clear dose-response correlation between birthweight and number of cigarettes smoked during pregnancy [29, 76, 82]. Roquer et al. [82] reported that weight reduction for term newborns was 458 g between mothers smoking more than 10 cigarette/day compared to those who neither smoked or were passively exposed to tobacco smoke ($p<0.001$). Moreover, not only maternal cigarette smoking but also environmental cigarette smoke exposure causes adverse pregnancy outcomes including fatal growth retardation and preterm delivery that lead to low birthweight [107, 117-119]. The weight reduction among newborns whose mothers were passively exposed to tobacco smoke was about 192 g [82].

1.5.1.2 Timing of cigarette smoke exposure

Fetal length increases most rapidly towards the end of the second trimester, while weight gain primarily occurs during the third trimester [20, 85]. Therefore, maternal cigarette smoke exposure during those periods would be critical for inhibiting fetal growth. Lieberman et al. [30] demonstrated that women who continue to smoke during pregnancy have a two fold increased

risk of giving birth to undersized infants than were nonsmokers. No association with IUGR was observed for women who quit smoking before the third trimester. On the other hand, women who began smoking during the second or third trimester of their pregnancy had a rate of small-for-gestational-age birth close to that for women who smoked throughout pregnancy [30].

Lin [20] and Heins et al. [4], however, suggested that fetal growth retardation could occur at a much earlier stage with involvement of genetic abnormalities and other teratogenic effects. Active and passive cigarette smoke exposure might result in significant genotoxic and chromosomal damage to the embryo that affect fetal growth in later gestational stages. Lin [20] suggested that the interaction and combination of risk factors including maternal behavior, medical and obstetrical factors, and stage of gestation could be the important factors of progression of fetal growth retardation.

1.6 Measurement of cigarette smoke exposure

A large number of epidemiological studies have examined the association between maternal cigarette smoking and low birthweight utilizing self-reported cigarette smoke exposures. However, several studies have suggested the presence of self-reporting bias. Etter et al. [120] had provided misclassification rates for current cigarette smokers who reported as non-smokers. Misclassification rates tend to be small but the rates are found to be different by ethnicity. The rates of minority groups are more likely misclassified.

Biochemical assessment gives much clearer indications of the effects of cigarette smoke exposure on physiological parameters and reduces misclassification. Cotinine, which is one of

the primary metabolites of nicotine, has been widely used as a biomarker for assessment of active or passive exposure to cigarette smoke. Single and also multiple time point cotinine measurement studies have been done to demonstrate a more clear association between maternal cigarette smoking and also passive smoke exposure and fetal growth retardation [121-124]. In addition, cotinine can be detected in cord blood and its concentrations are related to quantity of daily maternal smoking and passive cigarette smoke exposures during pregnancy [125, 126]. Cord serum cotinine appears to be the most adequate biomarker of fetal exposure to tobacco smoke at the end of pregnancy, distinguishing not only active smoking from passive smoke exposure but also exposure to environmental tobacco smoke from non-exposure [126].

1.6.1 Cotinine

Cotinine levels are a widely used biomarker for detecting active and passive cigarette smoke exposures. However, there are wide interindividual differences in nicotine-cotinine metabolism that are mainly mediated by the CYP2A6 metabolic enzyme. CYP2A6 is predominantly an hepatic enzyme with some expression observed in specialized extrahepatic cell types. Nicotine and a few other tobacco specific nitrosamines are metabolized by this enzyme. Nicotine is first oxidized to nicotine iminium ion by CYP2A6 and subsequently to cotinine by cytosolic aldehyde oxidase. In this reaction, up to 80% of nicotine is metabolized to cotinine [127].

Recently, a number of genetic polymorphisms in this gene have been identified which influence the enzyme activities and characteristics. Yang et al. [128] has reported that a *CYP2A6* polymorphism which results in loss of enzyme activity was significantly associated with the

urinary cotinine level. Therefore, *CYP2A6* polymorphisms may modulate the amount of nicotine/cotinine exposure to individuals and may affect adverse pregnancy outcomes.

Cotinine is a sensitive and specific biomarker for cigarette smoke exposure. However, it has very short lifetime (48 h). Thus, it is not an ideal marker to detect cigarette smoke exposure more than two days ago. It would be difficult to detect moderate smokers who do not smoke daily. Moreover, cotinine level widely reflects based on the interval of time between the last exposure and measurement time. Cotinine levels may therefore produce false negative results of smoking status and/or the level of tobacco smoke exposures.

1.7 Tobacco specific carcinogens

Although more than 4000 chemical compounds have been identified in cigarette smoke, the biological activities of only a few hundred have been characterized [129]. Many tobacco smoke constituents are defined as promoters of the neoplastic process, some are mutagenic, some are neurotoxic, some cause or intensify allergies, and some are fetotoxic [86]. It is also well documented that the concentrations of most tobacco smoke constituents associated with serious health damaging properties are higher in side-stream than in mainstream smoke [107].

Based on types of tobacco, styles of smoking and sources of smoke such as mainstream smoke and sidestream smoke, the composition of chemical compounds contained in smoke are different [107]. These hazardous substances are qualitatively similar in both mainstream and sidestream, although quantitatively, sidestream contains much more of almost each substance. The quantitative difference between chemical constituents of these two types of smoke

represents a factor between 2 to a few hundred [107]. Table 3 summarizes a partial listing of known carcinogens/mutagens contained in mainstream and sidestream smoke.

Table 3. Partial list of toxic compounds contained in mainstream and sidestream tobacco smoke [107]

Compounds	Examples	Carcinogenicity	Unit	Mainstream	Sidestream
Aromatic amines	Aniline	+	ng/cigarette	64	10800
	o-Toluidine	+		162	3030
	ß -Naphthylamine	+		1.7	67
	4-Aminobiphenyl	+		4.6	143
Dioxins	Polychlorinated dibenzodioxins	+	pg/pack	770	1360
	Polychlorinated dibenzofurans	+		720	1670
Heavy metals	Cadmium	+	ng/cigarette	100	360
	Nickel			20	600
Nitrosamines	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone	+	ng/cigarette	100	400
	N-Nitrosodimethylamine	+		10	1000
	N-Nitrosopyrrolidine	+		6	180
Polycyclic aromatic hydrocarbons	Anthanthrene	+	ng/cigarette	22	39
	Benz0[a]fluorine	+		184	751
	Benzo[a]pyrene	+		44	199
	Chrysene/Benzanthracene	+		191	1224
	Dibenz[a,j]anthracene	+		11	44
Radioactive elements	Polonium-210		pCi/cigarette	0/5	1.8
Solvents, gases	Acrolein		µ g/cigarette	60	480
	Ammonia			100	17000
	Benzene	+		12-48	120-480
	Carbone monoxide			20000	80000
	Formaldehyde	+?		70	700
	Toluene			160	960

1.7.1 Involvement of carcinogenic/mutagenic substances to the development of fetal growth retardation and preterm delivery

A number of studies have reported that cigarette smoke specific adducts and metabolites were detected in placental samples and even in urine from the fetus [80, 130-135]. Thus,

prenatal cigarette smoke exposure could be a significant risk factor associated with DNA damage levels that might lead to teratogenic events and other adverse reproductive outcomes. Several studies have reported that high frequencies of genetic abnormalities were observed in the fetus with growth retardation and prematurity [21-23].

There is increasing evidence from animal models that administration of tobacco related carcinogens/mutagens during pregnancy elevates the frequency of fetal genetic damage and future tumor formation. The presence of aromatic DNA adducts in the placentas of mice exposed to environmental carcinogens predicted the presence of adducts in fetal tissues, including liver, brain, heart, lung, kidney, intestines, and skin [136]. Lu et al. reported similar results [137] showing that higher levels of polycyclic aromatic hydrocarbon (PAH)-DNA adducts were observed in fetal tissues in early gestation compared to late gestation in a non-human primate model. Therefore, a fetus in the early gestational stages could be more vulnerable to the toxic effects of these compounds than a fetus in later gestational stages.

In humans, many xenobiotic agents including carcinogens/mutagens and their metabolites can pass through placenta and reach the fetus. Tobacco specific carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, (NNAL) and its metabolites, 4-[(methylnitrosamino)-1-(3-pyridyl)but-1-yl]β-*O*-D-glucosiduronic acid (NNAL-Gluc) have been detected in the urine of newborns whose mother smoked cigarettes [130]. Moreover, NNAL was detected even at early stage of pregnancy in the fetus [131].

In a recent molecular epidemiological study, Evans et al. [138] reported a dose-response relationship between maternal cigarette smoking and tobacco specific DNA adduct levels in the placenta. In addition, the levels of those DNA adducts were inversely associated with the birthweight. Moreover, Perera et al. [80] has reported a negative correlation between level of PAH–DNA adducts in cord blood cells and birthweight/head circumference. In addition, PAH-DNA adducts were significantly correlated with levels of chromosomal aberrations [139]. Interestingly, a significant association was found between low birthweight and childhood cancer (OR=2.5) and low birthweight was significantly related with risk of two of the most common childhood cancers, leukemias (OR=2.7) and lymphomas (OR=4.7) [140]. These studies clearly provide evidence of a molecular link between exposures of carcinogenic/mutagenic substances and adverse reproductive outcomes including fetal growth retardation, preterm delivery and to cancer.

1.8 Biotransformation

All living organisms are constantly exposed to foreign chemicals. Xenobiotic biotransformation is the primary biological mechanism for eliminating these molecules and is catalyzed by metabolic enzymes for conversion to a water-soluble chemical compound(s). In general, xenobiotic biotransformation is accomplished by a limited number of enzymes with broad substrate specificities. Multiple metabolic enzymes are involved in the biotransformation process and multiple pathways may exist for a given substrate. It is well-known that wide inter-individual differences exist in the metabolism of these harmful chemical compounds which are influenced by several factors including age, health conditions such as stress and diseases, nutritional level, presence of other exposures, and genetic factors such as genetic polymorphisms

of phase I/II metabolic enzymes. The genetic polymorphisms result in altered enzymatic activities, substrate specificities and create significant differences in the rates of xenobiotic biotransformation among individuals. This variability among individuals in xenobiotic biotransforming enzymes and susceptibility toward certain health defects is a field of study known as pharmacogenetics [141] and extensive research has been done to identify candidate risk factors for specific health effects.

1.8.1 Phase I and II xenobiotic biotransforming enzymes

The reactions catalyzed by xenobiotic biotransforming enzymes are classified as two separate processes, which are called phase I and phase II. Phase I reactions involve hydrolysis, reduction and oxidation. These steps expose or introduce a functional group such as -OH, -NH2, -SH, or -COOH, to increase substrate hydrophilicity, and are typically mediated by CYP enzymes. Phase II biotransformation reactions include glucuronidation, sulfonation, acetylation, methylation, conjugation with glutathione and conjugation with amino acids. The cofactors for these reactions react with functional groups that are either present on the xenobiotic or are introduced during the phase I biotransformation step. Most phase II biotransformation reactions increase hydrophilicity, hence they greatly promote the urinary excretion of foreign chemicals [141]. In the process of biotransformation, carcinogenic/mutagenic compounds are converted to reactive intermediates by oxidation of CYP enzymes that bind to cellular components, including DNA, RNA, and protein. DNA is the most critical target that is associated with increased incidence of cancer in genetically susceptible individuals [142]. For example, PAHs and aromatic amines contained in cigarette smoke can form bulky DNA adducts that block DNA synthesis, resulting in DNA strand breaks. Although mutational events occur in many genes

from exposure to carcinogenic compounds, mutation occurring in a few specific genes could be most critical to neoplastic transformation. Proto-oncogenes, cellular oncogenes and tumor suppressor genes are involved primarily in cellular growth, signal transduction, nuclear transcription and regulation of cell cycle. Mutated proto-oncogenes, cellular oncogenes and tumor suppressor genes are the most commonly observed in neoplastic tissues sites and they accelerate the neoplastic process [142].

1.8.2 Pharmacogenetics

The majority of CYPs and phase II metabolizing enzymes are predominantly expressed in liver but they are also found in virtually all tissues. These metabolic enzymes vary in expression according to tissue, gender, and demonstrate specificity for both substrates and inhibitors.

Moreover, many of these enzymes are associated with genetic polymorphisms resulting in altered activity and/or substrate specificity. Wide inter- and intra-individual differences associated with the metabolizing enzymes are caused by both environmental and genetic factors. A polymorphism is defined as the least common allele occurring in 1% or greater of the population whereas mutations are rare differences that occur in less than 1% of the population [143-145]. A summary of various types of genetic polymorphisms which may influence the expression of gene products or the catalytic activity of an enzyme is indicated below. These include: 1) Nucleotide variations in the coding region of the gene resulting in amino acid substitution and altered enzyme activity or substrate binding; 2) deletions in the coding region leading to an inactive enzyme or absence of protein synthesis; 3) polymorphisms in the noncoding region affecting transcriptional control elements involved in basal enzyme expression

and induction; 4) variations in the polyadenylation signal of a gene affecting transcript half-life and thus the quantity of enzyme; 5) gene amplification increasing the quantity of enzyme and 6) complex interactions of polymorphic genes and/or their enzyme catalysis products [129].

Single nucleotide polymorphisms (SNPs) are single-base differences in the DNA sequence that can be observed between individuals in the population. SNPs are present throughout the human genome with an average frequency of approximately 1 per 1,000 base pairs [143, 144, 146]. Other types of genetic polymorphisms result from the insertion or deletion of a sequence of DNA. The most common type of insertion/deletion polymorphisms are variable numbers of repeated base or nucleotide patterns in a specific region [144]. Repeated base patterns range in size from several hundreds of base pairs, known as variable number of tandem repeats, to two, three or four repeated nucleotides known as simple tandem repeats. The genome-wide frequency estimates for simple tandem repeats average every 3-10 kb in the genome [144]. Another type of insertion/deletion polymorphism involves the presence or absence of Alu segments at a particular genetic location. Alu segments, named according to the restriction enzyme used to detect them, contain two sequences approximately 120-150 bases in length, separated by an A base-rich segment. Insertions of this type occur on average approximately every 3 kb [144].

Some of the nucleotide substitutions are silent (do not result in an amino acid substitution) and may therefore be considered insignificant. However, recent studies have shown that silent SNPs in gene coding regions yield allele specific mRNA variants that differ markedly

in structural folding that may alter splicing, processing, translational control, and/or regulation of the mRNA [147].

1.8.3 Phase I/II metabolic enzyme genetic polymorphisms and carcinogenesis

Drug metabolizing enzymes, which often display genetic polymorphisms, convert many tobacco carcinogens into DNA-binding metabolites in target cells and can thereby modulate intermediate effect markers such as DNA adducts and ultimately, the risk for cancer [129]. Table 4 provides a partial list of enzymes responsible for metabolizing carcinogens contained in cigarette smoke.

Table 4. Carcinogens in cigarette smoke and phase I/II metabolic enzymes responsible for their metabolism

Compound	Examples	Metabolizing enzyme
PAHs	Benzo[a]pyrene	*CYP1A1, 1A2, 1B1, 2C8, 2C9, 2C18, 2C19, 3A4, GSTM1*
	Benzo[a]anthracene	*GSTT1*
		CYP1A1, 1A2, 1B1, 2B6, 2C9, GSTM1
Aza-arenes	Quinoline	*CYP1A1, 1A2, 2E1, 3A, NAT2, GST, EH*
N-nitrosamines	NNN, NNK, NNAL	*CYP1A2, 2A6, 2B1, 2E1, 3A4*
Aromatic amines	4-ABP	*CYP1A1, 1A2, NAT*
N-heterocyclic amines	PhIP	*GSTA1, GSTP1, NAT*
Aldehydes	Acetaldehyde	*CYP1A2, 2E1, 4A2, ALDH2, GST*
Miscellaneous	1.3-butadiene	*CYP2A6, 2E1, GSTT1*

The majority of xenobiotic agents are metabolized by phase I/II metabolic enzymes in two steps, activation and detoxification. During the process of biotransformation, some chemical

compounds are activated and have carcinogenic/mutagenic potential. For example, benzo[*a*]pyrene, which is contained in cigarette smoke, is metabolized by CYPs such as *CYP1A1*, to ultimately form a bay region diol epoxide. Diol epoxides are well recognized as tumorigenic metabolites and form bulky DNA adducts. The diol epoxides may be further metabolized by a phase II metabolic enzyme such as epoxide hydrolase as a detoxification pathway. Thus, genetic polymorphisms of the metabolizing enzymes may affect the level of activated metabolites in the body.

Differences in metabolic activation and detoxification pathways of environmental agents are likely to be a major source of interindividual variation in the level of cancer early biological effect markers such as somatic cell mutation frequency. Many molecular epidemiological studies have been completed to evaluate the associations between metabolizing enzyme allelic variants and cancer susceptibility [129, 148-152].

1.9 Cytochrome P450

Cytochrome P450s (CYPs) are classified as phase I metabolizing enzymes and are responsible for the metabolism of a wide variety of drugs and xenobiotics, including those found in cigarette smoke and alcohol. The highest concentration of CYP enzymes is found in the liver endoplasmic reticulum, but CYP enzymes are present in various tissues. CYP enzymes in liver play an important role in determining the intensity and duration of action of drugs, and they also play a key role in the detoxication of xenobiotics. Moreover, CYP enzymes in both liver and extrahepatic tissues play important roles in the activation of xenobiotics to toxic and/or tumorigenic metabolites.

All CYP enzymes are heme-containing proteins. The heme iron in CYPs is usually in the ferric (Fe^{3+}) state. When reduced to the ferrous (Fe^{2+}) state, CYP can bind ligands. The complex between ferrous CYP and a ligand absorbs light maximally as 450 nm, from which cytochrome P450 derives its name.

CYP enzymes use O_2 and electrons to oxidize substrates. Oxidation together with conjugation reactions yield metabolic products that are usually more hydrophilic than the parent compounds and therefore more readily excreted from the body. To date, 74 CYP gene families have been described, of which 14 exist in mammals (Table 5).

Table 5. Existing human CYP families and their metabolic roles [153]

CYP family	Main function
CYP1	Xenobiotic metabolism
CYP2	Xenobiotic metabolism
CYP3	Xenobiotic metabolism
CYP4	Fatty acid hydroxylation
	Xenobiotic metabolism?
CYP5	Thromboxane synthesis
CYP7	Cholesterol 7a -hydroxylation
CYP8	Prostacyclin synthesis
CYP11	Cholesterol side-chain cleavage
	Steroid 11ß -hydroxylation
	Aldosterone synthesis
CYP17	Steroid 17a -hydroxylation
CYP19	Androgen aromatization
CYP21	Steroid 21-hydroxylation
CYP24	Steroid 24-hydroxylation
CYP27	Steroid 27-hydroxylation
CYP51	Sterol biosynthesis

1.9.1 *CYP1A1*

The *CYP1A1* gene product, aromatic hydrocarbon hydroxylase (AHH), catalyzes the first step in the conversion of many environmental carcinogens, such as benzo[a]pyrene in cigarette smoke, to their ultimate DNA-binding, carcinogenic form. Human CYP1A1 protein is composed of 512 amino acid residues, which is smaller by 12 amino acids than its rodent equivalent. Human CYP1A1 is considered to function primarily as an extrahepatic enzyme and

both mRNA and protein are detected at high levels in lung, lymphocytes, and placenta, in contrast to undetectable levels in most human livers examined [154].

1.9.1.1 *CYP1A1* genetic polymorphisms

The human *CYP1A1* gene is located on chromosome 15. Currently, nine *CYP1A1* genetic polymorphisms have been characterized and some of the SNPs may cause an amino acid change that leads to conformational changes of the protein (Table 6). However, the functional significance of many of these SNPs remains unknown. *CYP1A1*2A* contains a single nucleotide polymorphism in the 3' flanking region, and is closely linked in Asians and less so in Caucasians with another polymorphism in exon 7 (*CYP1A1*2C*). The exon 7 point mutation is located in a region that codes for part of the catalytic region of AHH, and the presence of both of these polymorphisms confers a significant 3-fold increase in the catalytic activity of the AHH enzyme [155]. The *CYP1A1*3* allele is African in origin and is located just beside the codon 462-Ile/Val mutation (*CYP1A1*2B*) near the heme binding region. The allele frequency of this polymorphism is 0.09 in African Americans and 0.13 in Africans [155]. In a recent study, there was not a significant association observed between the *CYP1A1*3* allele and lung cancer but there was a trend of increased *CYP1A1*3* frequency among the nonsmokers and moderately smoking cancer patients [156]. Many molecular epidemiological studies have been conducted to evaluate the association between *CYP1A1* genetic polymorphisms and specific types of cancer [129]. However, the results are currently inconclusive, mainly due to sample size and varying racial allele frequency differences.

Table 6. Known *CYP1A1* genetic polymorphisms

Allele	Position	Location	Enzyme activity
*CYP1A1*1A*	None		
*CYP1A1*1B*	-3219 C ? T		
*CYP1A1*1C*	-3229 G ? A		
*CYP1A1*2A*	3801 T ? C	3` flanking region	There is a linkage with *2C
*CYP1A1*2B*	2455 A ? G, 3801 T ? C		
*CYP1A1*2C*	2455 A ? G (Ile? Val)	Exon 7 in the heme-binding region	2 fold increase in microsomal enzyme activity
*CYP1A1*3*	3205 T ? C	Intron 7	
*CYP1A1*4*	2453 C ? A (Thr? Asn)	Exon 7 in the heme-binding region	
*CYP1A1*5*	2461 C ? A (R? S)		
*CYP1A1*6*	1636 G ? T (M? I)		

http://www.imm.ki.se/CYPalleles/cyp1a1htm [157]

CYP1A1 gene expression can be induced by many different carcinogenic compounds found in tobacco and cigarette smoke. Additional inducers of the *CYP1A* enzymes include charcoal-broiled meat, which is a source of polycyclic aromatic hydrocarbons, cruciferous vegetables, and omeprazole, a protein-pump inhibitor used to suppress gastric acid secretion.

1.9.2 *CYP2E1*

The CYP2E1 enzyme is known to be an ethanol-inducible enzyme, and is involved in the metabolism of many low molecular weight chemical compounds including alcohol, halogenated alkanes and other procarcinogenic substances including *N*'-nitrosonornicotine (NNN), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [158]. CYP2E1 is primarily expressed in human liver and possibly in the kidney, lung, and lymphocytes [159]. Wide interindividual difference exist in *CYP2E1* protein or activity levels ranging from 6-20 fold when induced by

alcohol consumption [158]. It has been suggested that the observed variation might in part, be genetically determined.

1.9.2.1 *CYP2E1* genetic polymorphisms

Several genetic polymorphisms in the upstream regulatory sequence and in certain introns of *CYP2E1* have been detected and their relationship to both *CYP2E1* activity and disease susceptibility have been widely investigated (Table 7). To date, five genetic polymorphisms have been characterized in the *CYP2E1* gene. A C to T transition PstI or RsaI polymorphism, located in the regulatory 5'-flanking region of the *CYP2E1* gene, has been identified (*CYP2E1*5B*). *In vitro* expression studies indicate that this variant allele is associated with increased transcriptional activity and it has been suggested that individuals heterozygous or homozygous for the variant have an increased susceptibility to alcoholic liver disease which would be consistent with higher than normal *CYP2E1* activity. Lack of the Rsa I restriction site is associated with higher transcriptional activity, protein levels, and enzyme activity than the wild-type allele [160]. The frequency of the *CYP2E1*5B* allele varies in different ethnic populations and is higher among Asians (0.19 – 0.27) but lower in Caucasian and African Americans (0.01 – 0.05). A number of molecular epidemiological studies have been performed to evaluate the association between this polymorphism and alcoholic liver disease and/or cigarette smoking-related hepatocarcinogenesis, but results have been inconclusive. Other genetic factors or other environmental factors such as diet may explain the large interindividual variations observed.

Table 7. Known *CYP2E1* genetic polymorphisms

Allele	Location	Known Effect
*CYP2E1*1A*	None	
*CYP2E1*1B*	9893 C ? G	
*CYP2E1*1C*	6 repeats in the 5` flanking region	
*CYP2E1*1D*	8 repeats in the 5` flanking region	Increase activity
*CYP2E1*2*	1132 G ? A	R ? H reduced
*CYP2E1*3*	10023 G ? A	V ? I
*CYP2E1*4*	4768 G ? A	V ? I
*CYP2E1*5A*	-1293 G ? C, -1053 C ? T, 7632 T ? A	
*CYP2E1*5B*	-1293 G ? C, -1053 C ? T	
*CYP2E1*6*	7632 T ? A	
*CYP2E1*7A*	-333 T ? A	
*CYP2E1*7B*	-71 G ? T, -333 T ? A	
*CYP2E1*7C*	-333 T ? A, -352 A ? G	

http://www.omm.ki.es/CYPalleles/cyp2e1.htm [157]

1.10 Phase II metabolic enzymes

Phase II enzymes such as glutathione S-transferase (GST) and N-acetyltransferase (NAT) conjugate electrophilic compounds producing non-toxic agents, which can be readily eliminated from the body. Absent or deficient phase II enzyme activities may result in poorer elimination of electrophilic carcinogens, particularly in the presence of very active electrophilic activation by phase I enzymes. Phase II reactions generally proceed much faster than phase I reactions. Therefore, the rate of elimination of xenobiotics whose excretion depends on biotransformation by CYP followed by phase II conjugation is generally determined by the slower phase I reaction.

1.10.1 *Glutathione S-transferase* (*GSTs*) genes

GSTs are one of the most important cellular detoxification systems and have evolved to protect cells against reactive oxygen metabolites. These enzymes catalyze the conjugation of

aliphatic aromatic heterocyclic radicals, epoxides, or arene oxides to glutathione conjugation. Reaction at the electrophilic center of these compounds occurs at the sulfur atom of the glutathione molecule [161]. The GSTs are expressed at high levels in liver and to date, seven distinct classes are identified which are α, μ, θ, π, σ, κ and ζ [162]. These classifications are based on the substrate specificity, chemical affinity, protein structure, amino acid sequence and behavior of enzymatic kinetics. By definition, the subunits in the different classes share less than 40% amino acid identity. Generally, the subunits within a class are up to 70% identical and can form heterodimers, whereas the subunits in different classes are only up to 30% identical, which appears to prevent dimerization of two subunits from different classes.

1.10.1.1 *GSTM1*

Five *GST* μ genes (M1-M5) located on chromosome 1p13 have been identified and all are polymorphic [163]. Human GSTM1 is expressed primarily in the liver and was first identified as the benzo[*a*]pyrene oxide-conjugating enzyme [164]. GSTM1 is also present in stomach, brain and other tissues while GSTM2-GSTM5 isoforms have been detected in extrahepatic tissues and cell lines [163].

In humans, three *GSTM1* alleles, *GSM1*0, GSTM1*A, GSTM1*B*, have been described. The *GSTM1*0* is a gene deletion that results in the absence of enzyme activity. The *GSTM1*A* and *GSTM1*B* differ by a C to G substitution at base position 534, resulting in an amino acid change at position 173 (lysine to asparagine) substitution which encodes monomers that form active homo- and heterodimeric enzymes [141, 165, 166]. In vitro data indicate similar catalytic

effectiveness of the homo- and heterodimeric enzymes resulting from these alleles [163]. The *GSTM1* allele frequencies observed in Caucasians from North Staffordshire, England and Berlin, Germany were as follows *GSTM1*A* (26.5%); *GSTM1*B* (11.8%); *GSTM1*A/*B* (4.3%); and GSTM1*0 (57.3%) [167]. Another study has reported that in the U.S. approximately 49–54% of Caucasians are of the null *GSTM1* genotype [161]. Comparisons of the homozygous deletion genotype (*GTM1*0*) with genotypes containing at least one *GSTM1*A* or *GSTM1*B* allele are of primary interest to molecular epidemiological studies [161]. Since the *GST* family is a major detoxification pathway, *GSTM1* null carriers are more susceptible to electrophile exposures [161].

1.10.1.2 *GSTT1*

Human GSTT1, whose gene is localized on chromosome 22q11.2, is expressed in erythrocytes, lung, kidney, brain, skeletal muscles, heart, small intestine, spleen, and colon mucosal cytosol [162, 168]. GST theta has some differences in the catalytic activity compared with the other GSTs. The presence of Ser-11 residue in place of the N-terminal tyrosine creates the unique substrate specificity of the theta class [157]. Two functionally different genotypes in *GSTT1* have been identified that consist of an entire gene deletion, resulting in the lack of active GSTT1-1 enzyme [161, 162]. Experiments on dichloromethane (DCM)-incubated erythrocytes from humans with different genetic backgrounds measuring the GSH-conjugation rate and the release of formaldehyde showed that humans with the null genotype are non-conjugators (NC). Humans with the null genotype lack the GSTT1-1 enzyme, and are unable to conjugate DCM with GSH. Among positive genotype variants, erythrocytes from heterozygotes show an intermediate ability of GSH-conjugation whereas erythrocytes from the homozygotes show the

highest rate [162]. The *GSTT1* null genotype frequency is highest among Chinese (64.4%), followed by Koreans (60.2%), African Americans (20-24%), and Caucasians in the U.S. (23.7%) [161, 162].

1.10.1.3 *GSTM1/GSTT1* and carcinogenesis

Normal or increased GST enzyme activity or levels may protect susceptible tissues from adduct formation and somatic mutations in DNA by facilitating the conjugation and subsequent elimination of electrophilic carcinogens and mutagens. Absent or deficient GST enzyme activity may result in poor elimination of electrophiles, particularly in the presence of very active electrophilic activation by phase I enzymes. GST deficiencies may therefore result in increased risk of somatic mutation, leading to tumor formation [161]. Based on this hypothesis, extensive molecular epidemiological studies have been done to demonstrate increasing risk modulated by *GSTM1* and/or *GSTT1* genetic polymorphisms.

As shown in Table 8, several substrates are known to be metabolized by GSTT1-1 and absence of GSTT1-1 causes several types of DNA damage observed in in vitro and also in in vivo studies. According to those studies, absence of GSTT1-1 increases susceptibility to DNA damage and ultimately to cancer and other adverse health events.

Table 8. Partial list of effects of GSTT1-1 mediated metabolites

Source of exposure	Related chemicals or metabolites	Effect of GSTT1 absence in various in vitro assays	Effect among *GSTT1* null genotype in human
Cigarette smoke	PAHs	Not metabolized	
	ethylene expoxide	↑ hemoglobin adducts in WHB	↑ SCEs in PBL of WHB
1,3-butadiene	3,4-epoxibutene	↑ SCEs in WHB lymphocytes	↑ CAs
Styrene	styrene-7,8-oxide	↑ SCEs in PBL from WHB (2)	not determined
Ethylene	ethylene epoxide	↑ Hemoglobin adducts in WHB	↑ SCEs, ↑ Hb adducts *
Metabolite of aflatoxin B1	exo-aflatoxin B1-8,9-oxide	↑ mutagenicity (1)	not determined

* = effect seen only in non smokers, (1) = mutagenicity increased in transgenic *Salmonella* strans expressing the rat *GSTT1* compared with the parental strain, (2) = comparison between *GSTT1* null and postive human subjects

Extensive molecular epidemiological studies have been done to evaluate the association of GSTs in cancer risk. In general, among Caucasians, the *GSTT1* null genotype was associated with slightly increased risks of tumors of the head, neck, oral cavity, pharynx, and larynx [162]. Some studies described the *GSTT1-GSTM1* double null genotype as the genotype conferring the highest risk, with a 2.7-fold increase as compared with the *GSTT1-GSTM1* double positive genotype [162]. A number of studies have been done to evaluate the association between the *GSTT1* polymorphism and lung cancer. However, the findings to date are inconclusive. Since the *GSTT1* null genotype is highly associated with basal cell carcinoma of the skin and with brain tumors, it would be intriguing to ascertain if both of these cancers have common causes, such as endogenous mutagenic substrates for GSTT1-1 [162]. Several studies have provided evidence to suggest that individuals lacking the GSTT1-1 enzyme might be at an increased risk of cancer of different organs whereas evidence that GSTT1-1 positive individuals have an increased risk of cancer to exposure to halogenated compounds are rather limited. In addition, *GSTT1* appears to be a possible risk factor in lung carcinogenesis when *GSTM1* is concurrently lacking, under particular circumstances and for a specific type of tumor, but its role remains to be

ascertained precisely [162]. Presumably these genotypes, alone or in combination, should identify subjects who are detoxification-deficient and consequently more likely to suffer formation of carcinogen-DNA adducts and/or mutations [169].

1.10.2 *NAT2**

N-acetyltransferase has a primary role in the activation and/or deactivation of many aromatic amine and hydrazine compounds. There are three N-acetyltransferase genes in humans, *NAT1*, *NAT2**, and a pseudogene (NATP), which all are located on chromosome 8p22. N-acetyltransferases are cytosolic enzymes found in the liver and many other tissues of most mammalian species. In humans, NAT1 is expressed in virtually all tissues, whereas NAT2 is expressed mainly in the liver and the intestinal tract. *NAT1* and *NAT2* share 87% nucleotide homology in the coding region, yielding 55 amino acid differences and have substrate specificity. *NAT2** is involved in the detoxification of several carcinogenic arylamines including ß -naphthylamine, 4-aminobiphenyl and in the bioactivation of food mutagens such as 2-amino-3-methylimidaz[4,5-*f*]quinoline [170]. This enzyme is highly polymorphic in humans and the presence of two germ line copies of any of several defective alleles of the *NAT2** gene produces a slow acetylation phenotype, leading to altered rates of metabolism of arylamines [170]. Currently, 26 alleles of the *NAT2** gene have been identified and the frequency varies widely among ethnic groups. Approximately, 50-60% of Caucasians have the slow acetylator phenotype but the worldwide distribution of this phenotype varies from 5% among Canadian Eskimos to 90% among Northern Africans [147]. *NAT2*4* is considered the wild-type allele,

however, *NAT2*4* is not the most common allele in many ethnic groups, including Caucasians and Africans.

The four most common alleles in Caucasian and African American population are *NAT2*5B, *6A, *7A* and **14A*. *NAT2*5B* (M1) and *NAT2*6A* (M2) account for over 90% of the alleles associated with slow acetylation among Caucasians [171]. The *NAT2*7A* (M3) is much more rare, (1-2% in Caucasians) and *NAT2*14A* (M4) which is African specific, occurs in approximately 9% of the African American population [170]. There is a bimodal distribution of acetylation activities and that a value of up to 0.6 for the 5-acetylamino-6-formylamino-3-methyluracil (AFMU)/1X ratio is a useful dividing point between slow and rapid acetylation phenotypes [170]. Bell et al. have demonstrated a 100% concordance between *NAT2** genotype and phenotype in Caucasian and African American populations [170]. The four most common alleles are summarized in Table 9.

Table 9. Selected *NAT2** genetic polymorphisms

Allel	Position	Amino Acid change(s)
NAT2*4	None	None
NAT2*5	341 T ? C	114 I? T
	481 C ? T	268 K? R
	803 A ? G	
NAT2*6	282 C ? T	197 R? Q
	590 G ? A	
NAT2*7A	857 G ? A	286 G? E
NAT2*14A	191 G ? A	64 R? Q

http://www.louisville.edu/medschool/pharmacology/NAT.html [172]

Extensive epidemiological studies have been done to investigate the role of *NAT2* polymorphisms in a number of cancers. Aromatic amines such as 4-aminobyphenyl and heterocyclic amines found in cigarette smoke and heterocyclic amines found in charcoal broiled meat produce tumors at a multiple sites in rodents but require metabolic activation to mutate DNA and initiate carcinogenesis [147]. Oxidation, the *N*-hydroxy-aromatic and *N*-hydroxy-heterocyclic amines are further activated by N-acetyltransferases to acetoxy intermediates, which react spontaneously with DNA to form DNA adducts [173].

Previous epidemiological studies have demonstrated that the slow acetylation phenotype is associated with higher risk for bladder cancer in textile dye exposed workers and with low risk for colorectal cancer. This reflects the specificity of the *NAT2** enzyme for N-acetylation of carcinogenic arylamines, a detoxification step, and O-acetylation of N-hydroxylated heterocyclic amines found in the diet, resulting in bioactivation.

1.11 Fetal metabolic enzyme activity

The majority of lipid-soluble xenobiotics including carcinogenic and mutagenic compounds can the reach fetus by placental transfer following maternal exposure [153]. Fetal biotransformation is known to be limited compared with adults but it is also well established that human fetal tissues metabolize many foreign compounds and endogenous substrates [174]. Since phase I/II metabolic enzymes are present at low levels at birth, and reach adult levels with 1-3 weeks of age, infants are considered highly susceptible to prenatal exposure to harmful substrates. Poor capacity for metabolic detoxification is probably one of the major reasons for the high sensitivity of the neonate to chemical carcinogenesis [175].

1.11.1 Phase I metabolic enzymes

Pelkonen [176] reported that the human fetal hepatic enzyme system is detectable at the age of 6-7 weeks of gestation, the end of embryogenesis. It has been demonstrated that several CYP metabolic enzymes such as CYP1A1, CYP1B1, CYP2C8, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7, are present in the fetal liver after 8 to 9 weeks of gestation [153]. In addition, significant xenobiotic metabolism occurs also before week 8 of gestation and some fetal extrahepatic tissues, most notably the adrenal gland, contain substantial levels of CYP enzymes [153]. At the age of 12-14 weeks, the enzyme system seems to attain a relatively constant level [176]. However, fewer forms of CYPs are present in the fetal liver compared with the adult liver, and their levels are generally lower. The amount of total CYP in fetal liver is approximately 0.2 to 0.4 nmol/mg microsomal proteins, which is 20 to 70% of the level in the adult liver [153].

The major CYP form in the human fetal liver is CYP3A7 and accounts for over 30% of the total fetal liver CYP content but it is only a minor form in the adult liver [153]. The nucleotide sequence of *CYP3A7* cDNA is approximately 90% similar to that of *CYP3A4*.

A recent study by Shimada et al. [177] identified the presence of very low levels of the CYP1A1 protein before 20 weeks of gestation and it is approximately one tenth of the adult liver levels. Hepatic *CYP1A1* mRNA has been detected in both the embryonic and fetal phase of development.

In early studies, CYP2E1, which is responsible for alcohol metabolism, was not believed to be expressed before birth [178], suggesting that some inhibitory factor restricts CYP2E1 expression in the intact liver [179]. However, more recent studies provide evidence for the expression of CYP2E1 in human fetal liver at gestational age from 16 to 24 weeks [180]. Moreover, Carpenter et al. detected ethanol oxidation in fetal liver microsomes and found it to being inhibited by an anti-CYP2E1 antibody. Immediately after birth, CYP2E1 levels increase dramatically due to a stabilization of the CYP2E1 protein, potentially a consequence of the increase in ketone bodies at this period. Thereafter, the levels of *CYP2E* mRNA and protein levels rise gradually [153].

1.11.2 Phase II metabolic enzymes

In 8 week embryos, GSTP1 was the predominantly expressed GST isoform in all tissue samples but the kidney in which GSTA is more highly expressed [181]. The relative levels of GST distribution observed was 23% *GSTA*, 73% *GSTP1* and 4% *GSTM1* [162, 165, 182]. The levels of *GSTM1* expression was comparable in all tissues studied. Levels of *GSTP1* were high in early gestation, but decreased during gestation, whereas *GSTM1* and *GSTA* expression were moderate and weak, respectively. Levels of *GSTT1* expression were not determined in these studies.

1.12 Prenatal Exposure and Preeclampsia Prevention (PEPP) study

Recent epidemiological studies have demonstrated that maternal environmental and/or occupational exposures, lifestyle factors and habits including cigarette smoking, alcohol

consumption and specific types of diets during pregnancy increased risk of subsequent infant and pediatric malignancies in human newborns [183-187]. Previous studies have found that maternal exposure to tobacco smoke and/or maternal lifestyle factors associated with lower socioeconomic status appeared to increase the frequency and alter the spectrum of the molecular mechanisms of somatic mutation and chromosomal aberration in utero [188]. It is also well established that those maternal lifestyle factors including cigarette smoke exposure affect the frequency of adducts observed in placental and also fetal DNA [132, 189, 190]. In addition, the level of chromosomal aberrations was modulated by maternal phase I/II metabolic enzyme genetic polymorphisms [191, 192]. It is also known that infants with growth retardation and/or premature births are more likely to have higher frequencies of adducts, DNA damages and chromosomal aberrations in the placenta and fetal cells.

Therefore, we hypothesized that, among mothers who were exposed to toxic substances such as tobacco smoke and alcohol during pregnancy, a panel of maternal susceptibility factors such as metabolic enzyme activities, maternal age, nutritional status, parity, stress levels, other genetic factors, related health condition, disease states and the presence of other exposures would influence the level of toxic substrates metabolized and reaching the placenta. Placental metabolism may further modulate the level of toxic metabolites which ultimately reach the fetus. It is also likely that the level of newborn DNA damage that may influence the incidence of adverse reproductive outcomes is modulated by fetal susceptibility factors including metabolic enzyme activities, and DNA repair capacity (see Figure 1).

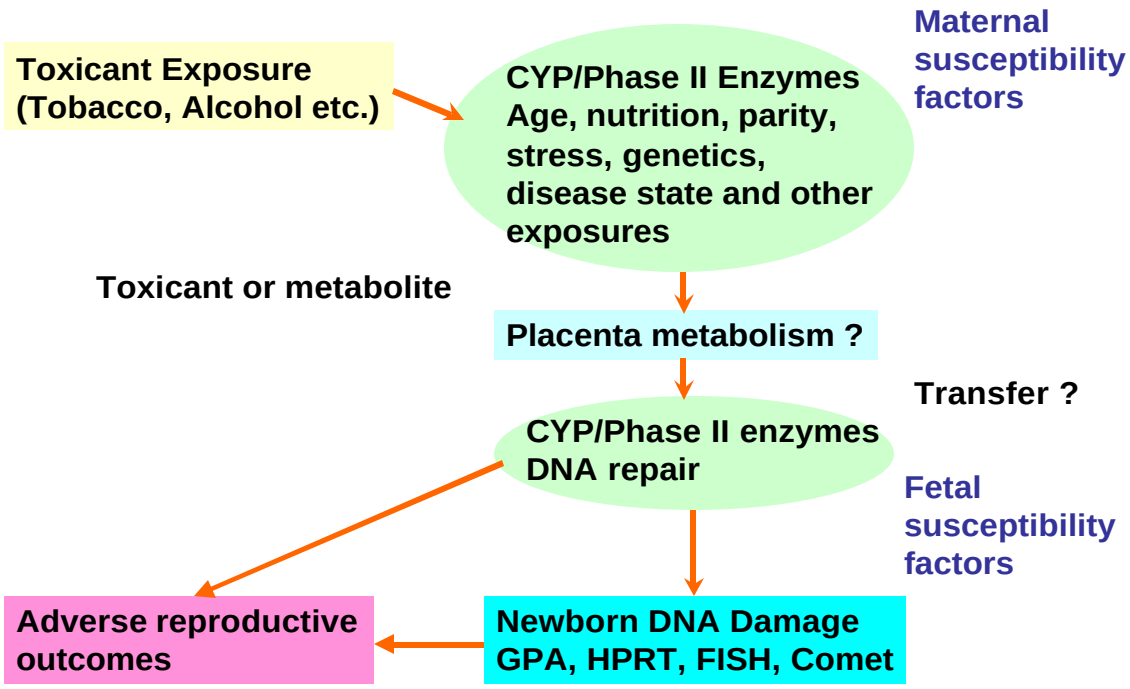

Figure 1. Model of environmental and genetic interactions that modulate newborn susceptibility to adverse effects

The Prenatal Exposure and Preeclampsia Prevention (PEPP) study at University of Pittsburgh/Magee Womens Hospital was designed as a molecular epidemiological study to investigate the potential transplacental genotoxic impact of prenatal exposures on levels of DNA damage observed in newborns and adverse reproductive outcomes by applying biomarkers of exposure, genetic susceptibility, and early biological effects. Table 10 lists the panel of the biomarkers evaluated in the PEPP study.

Table 10. Biomarkers evaluated in the PEPP study

	Biomarkers
Genetic susceptibility biomarkers [1]	Phase I/II metabolic enzyme genotypes (*CYP1A1, CYP2E1, GSTM1, GSTT1, NAT2**)
	Phase I metabolic enzyme mRNA (*CYP1A1, CYP2E1, CYP1B1*) expression
Tobacco smoke exposure biomarkers [1]	Serum cotinine
	4-Aminobiphenyl-hemoglobin (4-ABP Hb) adducts
Other early biological effect biomarkers [2]	*GPA* somatic cell mutation frequencies in erythrocytes
	HPRT locus mutation frequency and mutational spectra
	Single cell gel electrophoresis (SCGE)(Comet) assay
	FISH-based chromosome translocations

1 In collaboration with Drs. W. Bigbee and B. Day (University of Pittsburgh)

2 In collaboration with Drs. W. Bigbee, P. Keohavong, S. Grant (University of Pittsburgh) and Dr. J. Tucker (Lawrence Livermore National Laboratory)

This dissertation reports my investigation of the role of maternal and/or newborn phase I/II metabolic enzyme genetic polymorphisms contributing to increased risk of adverse reproductive outcomes including preterm delivery, fetal growth retardation and low birthweight in the PEPP population.

2 MATERIALS AND METHODS

2.1 Study population

The Prenatal Exposure and Preeclampsia Prevention (PEPP) project was designed as a multi-endpoint cohort study to evaluate the impact of maternal and/or newborn phase I/II metabolic enzyme genotype combinations in the presence of prenatal exposure to cigarette smoke, alcohol and other environmental compounds on the level of DNA damage observed in newborns, other effect biomarker endpoints and adverse reproductive outcome endpoints. The study population was recruited at the Magee-Womens Hospital (MWH), Pittsburgh PA and its associated private OB/GYN practice groups, starting in March 1997 (IRB #MWH-92-031).

Healthy pregnant women in the Pittsburgh area between the ages of 14 through 44 were recruited into the study. Those subjects at less than 18 weeks gestation at the time of recruitment and presenting for care at the Magee-Womens Hospital (MWH), or MWH associated OB/GYN practice groups were eligible for enrollment into the study. Exclusion criteria for the study include women presenting for their first prenatal visit or at any point up to 18 weeks gestation with chronic hypertension, defined as a blood pressure of greater than 140/90 mmHg, and women with a history of diabetes mellitus or known cardiac disease. All subject participants were given a baseline questionnaire by trained staff upon enrollment. The questionnaire included demographic characteristics such as age, ethnicity, education, marital status, household income, current employment status, occupation, occupational history and potential exposures and health insurance information. The questionnaire also included family history, medical history,

obstetric and gynecological history, anthropometric history, current medications, dietary intake and substance use and paternal smoking history. A follow up questionnaire, which focused on selected maternal lifestyle factors during the last three months of the pregnancy, was also administered to each study participant within 48 hours of delivery.

Birthweight was recorded from the medical chart and the gestational week was calculated from the last menstruation date, which patients had reported at the baseline interview and clinical visit. The number of gestational weeks were confirmed by uterus size measured by ultrasound.

2.2 Sample collection and processing

Maternal peripheral blood samples were collected at three times during the pregnancy; at less than 22 weeks (baseline), 28-32 weeks of gestation and one day postpartum. The blood samples collected at the first prenatal visit were classified as baseline. Umbilical cord blood samples were collected at delivery for newborn analysis. All peripheral and cord blood samples were drawn into a green top Vacutainer® tube (Becton Dickinson, Inc) containing sodium heparin anticoagulant by trained clinical staff and stored at -4°C. These peripheral blood samples were transferred to the Department of Environmental & Occupational Health research facility within 24 hours of collection. Both maternal and newborn blood samples were aliquoted in the receiving laboratory and were stored at -80°C until transfer to Dr. Romkes' laboratory. In order to ensure anonymity of the subjects and blind the laboratory investigations, a 6 or 9 digit unique identification number, Medical Record Number (MRN), was assigned to each subject and used to label blood samples, database entries, data printouts, and computer records.

For the Phase I and II metabolizing enzyme genotype analyses, 150 µl of maternal and/or newborn whole blood was used to isolate genomic DNA, either by the PureGene DNA Isolation Kit (Gentra Systems, Inc. Minneapolis, MN) or Instagene DNA isolation kit (Bio Rad, Hercales, CA). The extracted genomic DNAs were stored at –30°C until further analysis and all whole blood samples were stored at –80°C for long time storage.

2.3 DNA isolation

Two DNA isolation protocols, Instagene^{TM} Matrix DNA isolation kit (Bio Rad Company, Hercules, CA) and Puregene DNA Isolation Kit (Gentra Systems, Inc. Minneapolis, MN) were used. These two protocols are briefly described below.

2.3.1 Puregene DNA isolation kit

The protocol was adapted to a miniaturized form based on the recommended protocol from Gentra Systems. The frozen maternal peripheral blood and umbilical cord blood samples were thawed at room temperature for preparation of DNA isolation processing. Approximately 150 µl of whole blood was mixed with 450 µl of the RBC Lysis solution in a 1.5 ml microcentrifuge tube. The mixture was then vortexed for a few seconds and incubated at room temperature for 10 min. The mixture was inverted once during the incubation. After the incubation, the mixture was centrifuged for 20 seconds at high speed. The supernatant was removed with a pipette from the microcentrifuge tube leaving behind the visible white cell pellet

and about 10-20 µl of the residual liquid. The microcentrifuge tube was vortexed for few seconds to resuspend the cells in the residual liquid and 150 µl of the Cell Lysis Solution (Gentra System) and 0.75 µl of RNase A Solution (Gentra System) were added to the resuspended cells. The mixture was vortexed for few seconds and incubated in a 37°C waterbath for 15 min. The DNA isolation mixture was incubated at room temperature until all visible clumps were dissolved. The Protein Precipitation Solution (Gentra System) (50 µl) was added to the microcentrifuge tube and vortexed for 30 sec to mix with the cell lysate. Then, the microcentrifuge tube was centrifuged at 14,000 rpm for 6 min. The proteins precipitated on the bottom of the tube. The supernatant was poured into a sterile 1.5 ml microcentrifuge tube containing 150 µl of 100% isopropanol (Sigma). After a gentle mix, the tube was centrifuged at 14,000 rpm for 4 minutes. The supernatant was poured off and the microcentrifuge tube was drained briefly with clean paper. The white pellet deposited on the bottom of the microcentrifuge tube was washed with 150 µl of 70% ethanol (Sigma). The tube was centrifuged at 14,000 rpm for 4 min and the supernatant was poured off. The microcentrifuge tube was drained with clean paper. The white pellet was dried in a vacuum centrifuge for 5 min at the medium temperature setting. Fifty µl of the DNA Hydration Solution (Gentra System) was added to the microcentrifuge tube. The isolated DNA was incubated for complete hydration at room temperature overnight. The typical yield of genomic DNA using the PureGene DNA isolation kit with 150 µl of frozen whole blood was 200 ng/µl. The samples were stored at –30°C for phase I/II metabolic enzyme genotype analysis and –80°C for long time storage.

2.3.2 Instagene DNA isolation kit

The protocol was directly adapted from the Instagene recommended protocol. The frozen maternal/newborn whole blood samples were thawed at room temperature for DNA isolation processing. Twenty µl of whole blood was added to a sterile 1.5 ml microcentrifuge tube containing 1 ml of PCR dH_2O (Sigma). The mixture was incubated for 30 min at room temperature after a few seconds of vortexing. The mixture was centrifuged at 12,000 rpm for 3 min, and then the supernatant was removed from the microcentrifuge tube with a pipette leaving behind about 30 µl of the residual liquid. Instagene matrix (Bio Rad) (200 µl) was added to the microcentrifuge tube and the mixture was incubated for 30 min at 56°C. After the incubation, the microcentrifuge tube was vortexed for 10 sec and heated for 8 min at 100°C with a heat block. The mixture was vortexed for 10 sec after the heating and then centrifuged at 12,000 rpm for 3 min. The isolated genomic DNA was stored at –30 °C for the analysis and –80°C for long time storage. The genomic DNA isolated by Instagene DNA Isolation Kit needed to vortexed for few seconds and then centrifuged at 12,000 rpm for 3 min every time just before preparation of PCR analysis to increase PCR efficiency.

2.4 Phase I/II metabolic enzyme genotyping

Phase I (*CYP1A1*2A,*3* and *CYP2E1*5B*) and phase II (*GSTM1, GSTT1*, and *NAT2**) metabolizing enzyme genotypes were analyzed for 1,148 mother/newborn pairs by either Restriction Fragment Length Polymorphism – Polymerase Chain Reaction (RFLP-PCR) or differential PCR approaches. In general, the RFLP-PCR approach is commonly used for SNP detection and the differential PCR is useful for gene deletion detection analyses.

Briefly in RFLP-PCR, PCR is used to amplify a target DNA fragment. Figure 2 summarizes the basic principle of the PCR amplification. A single stranded DNA template is created by heating double stranded DNA near boiling temperature. The temperature is then lowered to allow specific primers to anneal to the complement DNA template. A single pair of forward and reverse primers is designed to recognize a complementary sequence surrounding the polymorphic site. Taq polymerase synthesizes new strands of DNA, complementary to the template, that extend a variable distance beyond the position of the primer binding site on the other template. The original and newly synthesized DNA stands are separated by increasing the temperature. New sets of primers are allowed to anneal to the newly synthesized DNA single strand template by reducing the temperature and Taq polymerase again synthesizes new strands. The PCR amplification process generates millions of copies of the target DNA region by repeating these cycles. Restriction enzymes can cleave the amplified DNA fragments due to the presence of a unique sequence in the fragments. If the DNA fragment carries a mutated nucleotide which creates a unique sequence recognized by a specific restriction enzyme, following enzyme digestion a different band pattern is observed from the fragment without the substituted nucleotide. Band patterns are detected by polyacrylamide gel electrophoresis for the determination of genotypes. For example, the *CYP2E1*5B* genotype was identified by gaining a recognition site for the restriction enzyme (Pst I) which results in two fragments. The expected band size for *CYP2E1 *5B/*5B* is 290 and 122 bp while the wild type genotype (*CYP2E1*1/*1*) band pattern is a single 412 bp fragment (Figure 3).

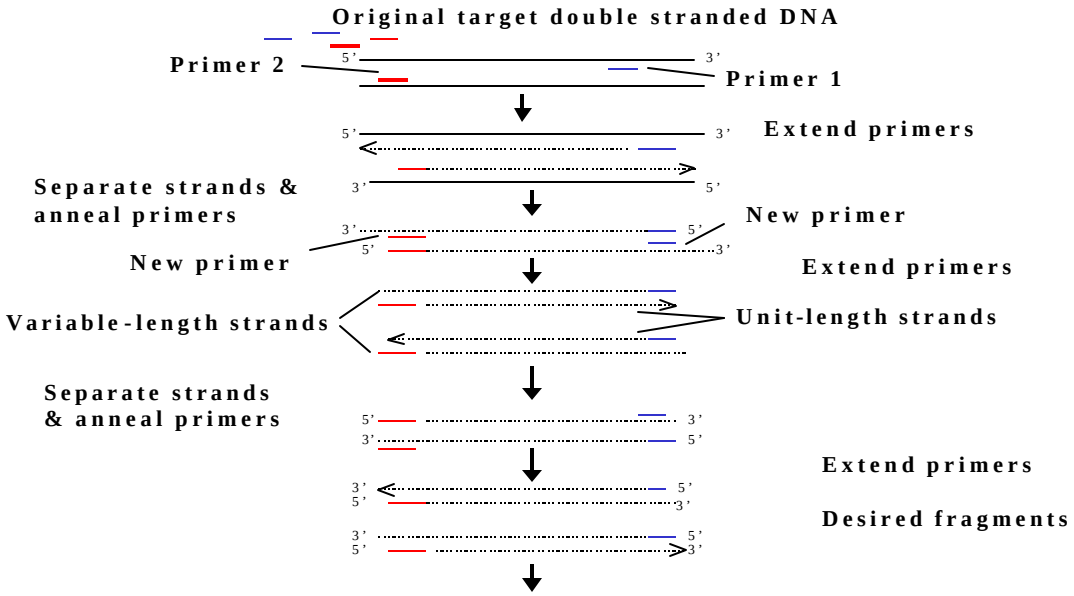

Figure 2. Polymerase chain reaction [193]

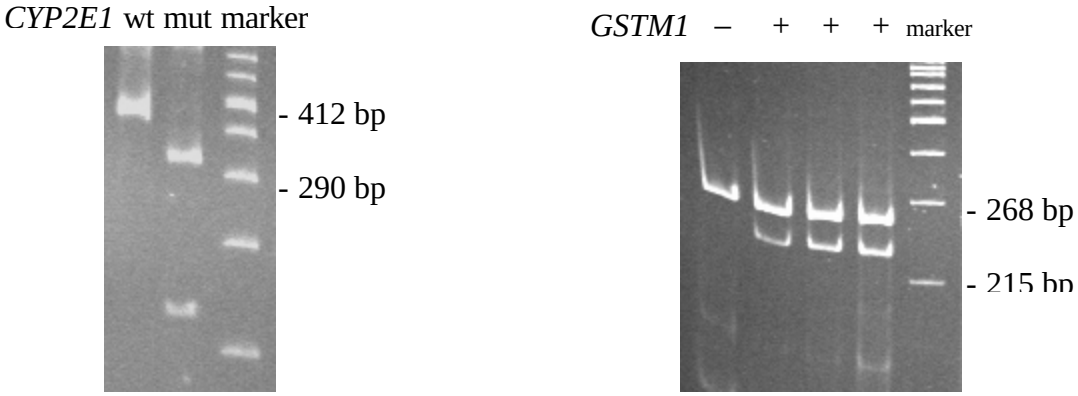

Figure 3. Genotype analysis for *CYP2E1* and *GSTM1*

Similarly for differential PCR, a set of forward and reverse primers are designed to anneal to template DNA strands within the polymorphic gene. If the gene is deleted, the primer cannot anneal to the template DNA to initiate new strand synthesis and the PCR amplification fails. As an internal control to confirm a successful PCR reaction, a house keeping gene such as

β-globin is amplified in the same reaction mix. As shown in Figure 3, the *GSTM1* null genotype appears as a single band at 268 bp (β-globin) but absent *GSTM1* gene (215 bp). On the other hand, the presence of two bands at the expected sizes represents the *GSTM1* positive genotype.

PCR conditions for the genotyping analyses are summarized in Table 11. Briefly, the PCR is carried out with dimethyl sulfoxide (DMSO) (Sigma), 1X PCR buffer (Perkin Elmer), $MgCl_2$ (Perkin Elmer), 10mM dNTPs (Pharmacia), and 400ng/sample of both forward and reverse specific primers (Midland). The amount of genomic DNA used in the analyses was dependent on the specific PCR reaction. The hot-start PCR reaction was initiated by a pre-PCR reaction by 5 min of denaturation (94°C) prior to the addition of AmpliTaq DNA polymerase (2.5 unit/reaction) (Perkin Elmer) at 86°C, followed by 2.5 min of extension at 94°C. Each PCR cycle had three different temperature cycles starting with 1) denaturation or the separation of genomic DNA to single strands at 94°C for 1 min, 2) an annealing step that allowed the primer to anneal at a specific temperature for 1 min and 3) then an extension step at 72°C for 3 min. The PCR cycles were repeated for 40 times followed by 7 min of extension at 72°C and a 4°C soak. PCR protocols for each specific genotype analysis were optimized and validated in Dr. Romkes' laboratory. Every PCR reaction was performed with at least one positive control sample of a known genotype confirmed by DNA sequencing as a test for reproducibility and a negative control used as a contamination indicator.

Table 11. PCR amplification conditions for phase I/II metabolic enzyme genotypes

Metabolic Enzymes	DMSO	1X Buffer	$MgCl_2$	Taq polymerase	Annealing Temperature	Reference
*CYP1A1*2A/*3*	Yes	Buffer II	1μM	AmpliTaq	55°C	[194]
*CYP2E1*5B*	Yes	Buffer II	1 μM	AmpliTaq	53°C	[195]
*NAT2**	No	Buffer F	No	AmpliTaq	57°C	[170]
GSTM1	Yes	Buffer II	1.5 μM	AmpliTaq Gold	55°C	[148]
GSTT1	No	Buffer B	No	AmpliTaq	55°C	[196]

For the RFLP analysis, the amplified PCR products were initially run on 8% poly-acrylamide (National Diagnostics) gels to verify that the PCR amplification was successful. PCR products were then digested with the appropriate restriction enzyme (Table 12) for approximately 2 hours at the recommended temperature in a waterbath. A stop solution, which contained formamide (Sigma), bromophenol blue (Sigma), and Tris Borate EDTA (TBE) buffer (Sigma) was added to the digested PCR products to terminate restriction enzymatic digestion. Then, the digested products were run on 8% poly-acrylamide gels at 300V for about 1 hour or until the stop solution reached the bottom of the gels. The gels were stained with ethidium bromide (Sigma) and photographed. Positive control samples representing the 3 possible genotypes were also digested with the restriction enzyme and run on each poly-acrylamide gel to verify 1) complete restriction enzyme digestion and, 2) accuracy of expected band pattern. A DNA fragment size ladder, 100 bp DNA (MBI Fermentas) was added to one of the lanes.

Table 12. Summary of phase I/II metabolizing enzymes, RFLP-PCR, differential PCR analysis protocols and expected band patterns

	Metabolic Enzymes	Allelic Variants	Expected Band Sizes (bp)	Restriction Enzyme	Reference
RFLP-PCR	*CYP1A1*	**1* (wt), **2A* (Msp), **3*	**1/*1*=1032 **1/*2A*=206,826,1032, **1/*3*=260,772,1032 **2A/*2A*=206,826 **3/*3*=260,772	MspI	[194]
	CYP2E1	**1* (wt), **5B*	**1/*1*=412 **1/*5B*=412,290,122 **5B/*5B*=290,122	PstI	[195]
	*NAT2**	**4* (wt),	**4/*4=1093*		[170]
		**5*(m1),	**5A/5B*= wt-660, 433 ht-1093, 660, 433 mut-1093	KpnI	
		**6A* (m2),	**6A*= wt-104, 122, 170, 317, 380 ht-104, 122, 170, 274, 317,380 mut-122, 274, 317, 380	TaqI/AvaII,	
		**7A* (m3),	**7A*= wt-811, 282 ht-1093, 811, 282 mut-1093	BamHI,	
		**14A* (m4)	**14A*= wt759, 189, 91, 53 ht-759, 280, 189, 91, 53 mut-759, 280, 53	MspI/Alu I	
Differential PCR	*GSTM1*	Present, null	β-globin band=268 (+C), *GSTM1*=215	None	[148]
	GSTT1	Present, null	β-globin band=268 (+C) , *GSTT1*=480	None	[196]

Since the *GSTM1* and *GSTT1* genetic polymorphisms are complete gene deletions, the differential PCR approach was performed. Briefly, the PCR reaction contained one set of forward and reverse primers for each gene, *GSTM1/GSTT1* and β-globin (a positive control). The primers were designed to amplify different fragment sizes of these genes which the expected fragment sizes summarized in Table 12. The amplified PCR products were then run on 8% poly-acrylamide gels to determine the band pattern. *GSTM1* or *GSTT1* positive, which has at least one copy of the gene, would be expected to show two bands of different band sizes and *GSTM1* or

GSTT1 null was determined as the presence of only β-globin band (Figure 3). Similarly, a known genotype positive control and a negative control were run with each gel for verification.

2.5 Quality control

A major concern in these analyses were the inclusion of a number of different quality control measures to monitor quality of data obtained. The strategies used for quality control are summarized below.

2.5.1 Lab positive control samples

Previously sequenced genomic DNA samples were used with every PCR analysis to verify reproducibility of RFLP-PCR and differential PCR reactions and to confirm accuracy of genotype classifications. Each possible allele combination for all genotypes screened was included in this positive control reference DNA sample set.

2.5.2 Protocol specific procedures for quality control

The entire DNA isolation protocol and PCR preparation were performed in separate chemical and laminar flow hoods, respectively. Only sterile filtered tips were used for the entire procedure and all microcentrifuge tubes were sterilized. All pipette tips were changed between samples to prevent cross-contamination. For PCR reaction setup, the hood, pipettes, tips, micro centrifuge tubes, 96-well PCR plates and other reagents needed for the PCR reaction except

primers and DNA polymerase were UV-irradiated for at least 5 min. This process is necessary to prevent the introduction of any foreign DNA to the PCR amplification process.

All PCR reactions were performed with at least one positive control, and a negative control (without DNA). These controls were included as the last tubes to be processed in order to detect the presence of any carryover contamination.

2.5.3 Genotype database specific procedures for quality control

Two individuals independently reviewed the polyacrylamide gel pictures for classification of genotypes. Following entry of the genotype data into the database, a second person cross-checked all results. The genotype analyses was repeated if the results were discordant.

2.5.4 Laboratory wide quality control

A monthly log documenting the calibration checks for the PCR thermal cyclers, balance, spectrophotometer, autoclave, pipettes, waterbaths and centrifuges was maintained. The pipettes were calibrated every 6 months by an external professional service.

2.6 Data entry and analysis

The maternal/newborn phase I/II metabolic enzyme genotypes, maternal demographic characteristics and lifestyle variables were entered in an Excel spreadsheet (Microsoft). All

statistical analyses were performed using STATA version 7 statistic software (Stata Corporation).

2.6.1 Categorization of phase I/II metabolic enzyme genetic polymorphism genotypes

Initial characterization of the genotype allelic frequency data for the following metabolizing enzymes were classified according to predicted phenotypic activity, from which the high risk (value=1) or low risk groups (value=0) were derived. If the maternal or newborn subject carried at least one copy of *CYP1A1*2A* or *CYP1A1*3*, the subject was classified as risk group (value=1). On the other hand, if the subject carried two copies of *CYP1A1*1* (wild type), they were classified as low risk group (value=0). Similarly, if the subject carried at least one copy of *CYP2E1*5B*, they were classified as the high risk group (value=1). If the subject carried two copies of *CYP2E1*1* (wild type), they were classified as the low risk group (value=0). For both *GSTM1* and *GSTT1* genotype classifications, the subject was categorized in the high risk group (value=1), if they had the null or absent genotype. If they carried at least one copy of the gene, they were classified as the low risk group (value=0). Finally for the *NAT2* genotype, individual subjects were categorized as a slow acetylator if they carried at least two copies of *NAT2*5A/5B*, *NAT2*6A*, *NAT2*7A* or *NAT2*14A* or a fast acetylator if they carried no or one copy of the *NAT2** allelic variant for the high risk group (value=1) and for the low risk group (value=0), respectively.

2.6.2 Classification of low birthweight, preterm delivery and small for gestational age

From among the overall study group, singleton live birth maternal/newborn pairs (n=1148) were classified into five groups based on birthweight and gestational weeks as follows: 1) preterm delivery (n=86); 2) low birthweight (n=93); 3) preterm delivery and low birthweight (n=53); 4) small for gestational age (SGA) (n=107); and 5) average for gestational age (AGA) (n=948). Preterm delivery was defined as less than 37 gestational weeks. Low birthweight was defined as weight less than 2,500 g. Group #3 included both of these criteria of low birthweight and preterm delivery. SGA was defined as weight at or below the 10^{th} percentile of birthweight for gestational age at full term (≥ 37 weeks) delivered at MWH. AGA was defined as ≥2,500 g in birthweight at full term.

2.6.3 Definition of maternal risk group variable in the last trimester

Maternal risk groups in the last trimester were calculated and classified according to the method developed by Dr. Gordish in her dissertation [197]. Briefly, the risk groups consisted of four separate maternal risk factors, which were maternal cigarette smoking, alcohol intake, consumption of caffeine and charbroiled meat consumption in the last trimester. These four risk factors were combined into one categorical risk group variable as follows. If the maternal subject smoked at least one cigarette per day during the last trimester, she was classified as positive in maternal cigarette smoke category. If the maternal subject had not smoked or had less than one cigarette per day, she was classified as negative in this risk category. Similarly, if maternal subject drank any alcohol during the last trimester, she was classified as positive and if she had not, she would be negative in the alcohol intake risk category. Daily caffeine

consumption was determined from four-caffeinated beverage intake levels, which were regular coffee, black tea, soft drink and hot chocolate, during the last trimester. The maternal subject was classified as positive for caffeine consumption, if she had at least one caffeinated beverage per day. For the consumption of charbroiled meat risk category, the maternal subject was classified as positive if she ate charbroiled meat once or more per month. All four risk categories were then combined into one categorical risk group variable comprising three risk groups. Those maternal subjects classified as negative for all four risk factors were placed in the low risk group (value=0). Maternal subjects classified as positive for any one or two of the risk categories were placed in the medium risk group (value=1) and finally, maternal subjects classified as positive for any three or four of the risk categories were placed in the highest risk group (value=2).

2.6.4 Univariate analyses

Univariate comparisons between the birthweight/gestational week and specific maternal/newborn characteristics/lifestyle factors were performed. These maternal factors included maternal age, household income, maternal education level, maternal cigarette smoking, passive cigarette smoking, alcohol intake, caffeine consumption, charbroiled meat intake, maternal third trimester risk group and maternal and/or newborn's phase I/II metabolic enzyme genotypes. These univariate analyses were performed by single variable logistic regression and also by single variable linear regression.

2.6.5 Multiple regression analyses

Both logistic and linear multivariable regressions were performed to determine maternal and/or newborn risk factors associated with lower birthweight and/or preterm delivery. In logistic regression, the adverse reproductive outcome, low birthweight and/or preterm delivery were the dependent variables. In the linear regression models, birthweight was the dependent variable. The independent variables such as maternal age, household income, maternal education level, maternal cigarette smoke in the last trimester, passive cigarette smoke exposure, alcohol intake, caffeine consumption, charbroiled meat intake, maternal third trimester risk group and maternal and/or newborn phase I/II metabolic enzyme genotypes were tested for an association with low birthweight and/or preterm delivery. Once maternal demographic and lifestyle risk factors were identified, maternal and newborn genetic polymorphisms of phase I/II metabolizing enzymes were tested for modulation of these adverse reproductive outcome.

3 RESULTS

3.1 Maternal demographic characteristics

The study population consisted of 1148 maternal/newborn pairs of which, by self-report, 376 (32.8%) were African American, 754 (65.7%) were Caucasian and 18 (1.5%) were defined as other ethnic group (Table 13). Maternal age ranged from 13 to 45 years old and African American women were significantly younger than women in the other ethnic groups ($p<0.001$). The mean maternal age was 22.5 ± 5.1 years in African Americans, 26.4 ± 6.3 years in Caucasians, and 27.4 ± 6.0 years in others. More than 40% of the African American women were under age 20 years old, while approximately 20% of women in Caucasian and 15% of women in other ethnic groups were in this young maternal age group.

The family income profiles for African American women tended to be lower compared to Caucasians and others. However, the differences in mean income were not statistically significant ($p=0.20$) at $\alpha=0.05$. There are distinct differences in family income among racial groups, especially in the lowest and highest income groups. Almost a two-fold higher percentage of African American women (33.0%) were in the lowest income group (<$10,000) compared with Caucasian women (18.3%). Similarly, a more than three fold higher percentage of Caucasian women (36.4%) and also women in the other racial groups (27.8%) were in the highest income group (≥$35,000) compared with African American women (9.0%). In addition, approximately a two to three times higher percentage of African American women (30.1%) did

not know their family income compared with Caucasian women (10.5%) and women of other ethnic groups (16.7%).

Maternal education level demonstrated a similar pattern to that of family income with significant differences among ethnicity ($p<0.001$). African American women were more likely to have a lower level of education compared with women in the other racial groups. Approximately a two fold higher percentage of African American women (29.3%) had not graduated from high school compared with Caucasian women (14.9%) and the other ethnic group women (11.1%). Moreover, more than a five fold higher percentage of Caucasian women (28.9%) and fourteen fold for women in the other ethnic groups (61.1%) were college graduates or higher compared with African American women (4.2%).

Table 13. Maternal demographic characteristics in the study population

	African American N=376 (32.8%) Mean ± s.d. (range)	Caucasian N=754 (65.7%) Mean ± s.d. (range)	Other N=18 (1.5%) Mean ± s.d. (range)	Overall N=1148 Mean ± s.d. (range)
Maternal age *	22.5 ± 5.1 (13-41)	26.4 ± 6.3 (14-45)	27.4 ± 6.0 (17-37)	25.1 ± 6.2 (13-45)
≤ 15 years old	7 (1.9%)	4 (0.5)	0	11 (1.0%)
16-20 years old	158 (42.0%)	157 (20.8%)	3 (15.7%)	318 (27.7%)
21-30 years old	179 (47.6%)	379 (50.3%)	9 (50.0%)	567 (49.4%)
31-39 years old	30 (8.0%)	196 (26.0%)	6 (34.3%)	232 (20.2%)
≥40 years old	2 (0.5%)	18 (2.4%)	0	20 (1.7%)
Family income:				
<$10,000	124 (33.0%)	138 (18.3%)	4 (22.2%)	266 (23.2%)
$10,000-<$20,000	67 (17.8%)	145 (19.2%)	4 (22.2%)	216 (18.8%)
$20,000-<$35,000	38 (10.1%)	117 (15.5%)	2 (11.1%)	157 (13.7%)
≥$35,000	34 (9.0%)	275 (36.4%)	5 (27.8%)	314 (27.4%)
Don't know	113 (30.1%)	79 (10.5%)	3 (16.7%)	195 (16.9%)
Maternal education*				
< 12 grade	110 (29.3%)	112 (14.9%)	2 (11.1%)	224 (19.5%)
High school diploma	148 (39.4%)	260 (34.5%)	2 (11.1%)	410 (35.7%)
Some collage	102 (27.1%)	164 (21.7%)	3 (16.7%)	269 (23.4%)
BS or greater	16 (4.2%)	218 (28.9%)	11 (61.1%)	245 (21.4%)

* Indicates a statistically significant difference among ethnic groups (p = 0.05).

3.2 Maternal smoking history, alcohol intake and charbroiled meat consumption at enrollment

3.2.1 The validity of self-reported smoking

Plasma cotinine levels were measured in order to evaluate the reliability of the maternal self-reported cigarette smoking in the last trimester for a subset of randomly selected 107 subjects. Maternal whole blood samples were collected when the subjects were admitted to MWH for delivery. Positive tobacco exposure was defined as a cotinine levels of ≥25 ng/ml indicative of active smoking [124, 198]. The statistical analyses of the degree of agreement

between self-reported active smoking and cotinine level were performed by Dr. Gordish[197]. According to her analysis, of the 30 women who reported smoking during the last trimester, 24 had cotinine positive tobacco exposure while 6 did not. Of the 77 women who reported no smoking exposure during the last trimester, 73 had no cotinine verified exposure while 4 showed positive cotinine levels. These observations yielded a sensitivity value of 85.7% and a specificity value of 92.4%. The level of agreement calculated by the kappa statistic was 90.7%. Thus, cigarette smoking self-reports among the PEPP study population were considered reliable.

3.2.2 Smoking history at enrollment

More than 50% of women in the study population had smoked at least 100 cigarettes in their lifetime (44.4% of African Americans, 62.6% of Caucasians and 33.3% of other racial groups). Approximately 46% of the women were current smokers not baseline, defined as smoking one year prior to the pregnancy (40.2% of African Americans, 49.7% of Caucasians, and 33.3% of other racial groups). There was a significant difference in the number of cigarettes smoked per day among racial groups (p=0.009) (Table 14). Caucasian women tended to be heavier smokers compared with women in other racial groups. The mean number of cigarettes smoked per day was 16.3 ± 9.1 in Caucasians, 10.3 ± 7.6 in African Americans and 10.2 ± 8.2 in the other ethnic groups. There was also a significant difference in the amount the women smoked prior to pregnancy among racial groups (p=0.001). More than 66% of women in all racial groups continued to smoke after they became pregnant (64.9% of African Americans, 67.2% of Caucasians and 66.7% of others). The mean number of cigarettes smoked per day decreased substantially compared with the mean prior to pregnancy, 6.2 ± 9.3, 8.4 ± 6.8, and 3.5

± 1.7 cigarette per day, respectively. There are significant differences among racial groups for the mean number of cigarette smoked once pregnant (p=0.001). However, approximately 10% of smokers did not change the amount smoked since pregnant, 10.6%, 10.4% and 0%, respectively.

3.2.3 Alcohol intake and charbroiled meat consumption at enrollment

The vast majority of women consumed alcoholic beverages prior to pregnancy. However, there was a significant difference in the number of drinkers ($p<0.001$) and the mean grams of alcohol consumed per week ($p<0.001$) among racial groups. The amount of alcohol (grams) was calculated as follows. First, the numbers of drinks per week for each type of alcoholic beverage (beer, wine and liquor) were determined separately. These values were converted to grams of alcohol where one can of beer (12oz) contained 12.8 g of alcohol, one grass of wine (4oz) contained 10g of alcohol and one shot of liquor (1.5oz) contained 15.1g of alcohol.

Caucasian women were more likely to drink and consumed higher amounts of alcohol per week compared with African American women and other racial groups. The average grams of alcohol consumed per week was 61.0 ± 128.0 in African Americans, 73.0 ± 142.3 in Caucasians and 31.3 ± 78.5 in the other ethnic groups. Only 10.5% of women continued to drink once pregnant (9.0% of African Americans, 11.5% of Caucasians and 0% of other).

The consumption of charbroiled meat was also significantly different among racial groups ($p<0.001$). African American women consumed less charbroiled meat compared with Caucasian women and others. Over 50% of African American women consumed charbroiled meat less than once per month or never while 39.7% in Caucasians and 38.9% of others reported such intake.

Table 14. Maternal smoking history, alcohol intake and charbroiled meat consumption at enrollment

	African American N=376 (32.8%) Mean ± s.d. (range)	Caucasian N=754 (65.7%) Mean ± s.d. (range)	Other N=18 (1.5%) Mean ± s.d. (range)	Overall N=1148 Mean ± s.d. (range)
Smokers (N)	167 (44.4%)	472 (62.6%)	6 (33.3%)	645 (56.2%)
Current smokers (N)*	151 (40.2%)	375 (49.7%)	6 (33.3%)	532 (46.3%)
Cigarettes/day prior to pregnancy *	10.3 ± 7.6 (1-45)	16.3 ± 9.1 (0.1-50)	10.2 ± 8.2 (0.3-20)	14.5 ± 9.1 (0.1-50)
Continue to smoke since pregnant	98 (64.9%)	252 (67.2%)	4 (66.7%)	354 (66.5%)
Cigarettes/day since pregnant*	6.2 ± 9.3 (0.2-80)	8.4 ± 6.8 (0.1-40)	3.5 ± 1.7 (1-5)	7.6 ± 7.6 (0.1-80)
Quit smoking	53 (35.1%)	123 (32.8%)	2 (33.3%)	178 (33.4%)
Increased smoking	6 (4.0%)	4 (1.1%)	0	10 (2.0%)
Decreased smoking	76 (50.3%)	209 (55.7%)	4 (66.7%)	289 (54.3%)
No change in smoking	16 (10.6%)	39 (10.4%)	0	55 (10.3%)
Drinkers (N)	272 (72.3%)	692 (91.8%)	15 (83.3%)	979 (85.3%)
Current drinkers (N)	236 (62.8%)	630 (83.6%)	14 (77.8%)	880 (76.7%)
Drinks/week prior to pregnancy (g)	61.0 ± 128.0 (0.1-1177)	73.0 ± 142.3 (0.1-1281)	31.3 ± 78.5 (0.6-296.4)	69.1 ± 137.9 (0.1-1281)
Continue to drink once pregnant	34 (9.0%)	87 (11.5%)	0	121 (10.5%)
Charbroiled meat				
Never	51 (13.6%)	82 (10.9%)	3 (16.7%)	136 (11.9%)
< 1x/month	182 (48.4%)	217 (28.8%)	4 (22.2%)	403 (35.1%)
1-4X/month	94 (25.0%)	301 (39.9%)	6 (33.3%)	401 (34.9%)
>4x/month	37 (9.8%)	132 (17.5%)	4 (22.2%)	173 (15.1%)
No response	12 (3.2%)	22 (2.9%)	1 (5.6%)	35 (3.0%)

* Indicates a statistically significant differences among racial groups

3.3 Maternal cigarette smoking and alcohol consumption during the third trimester of pregnancy

Approximately 57% of smokers continued to smoke during the last trimester of pregnancy (58.3% of African Americans, 56.8% of Caucasians, and 66.7% of other ethnic groups). Overall, approximately 27.7% of the mothers in this study population who smoked during the last trimester (24.1% of African Americans, 29.5% of Caucasians and 25.0% of other ethnic groups). The mean number of cigarettes smoked per day was significantly different among racial groups ($p=0.003$). Among Caucasian women, the mean number of cigarettes per day was 9.0 ± 6.9, while women in other racial groups smoked less (6.4 ± 7.6 of African American and 3.3 ± 2.6 of others).

More than a three fold higher percentage of Caucasian women consumed alcohol in the last trimester (11.5%) while only 3.8% of African American women reported drinking. This difference was statistically significantly different ($p<0.001$). The average grams of alcohol consumed in the third trimester was 5.9 ± 6.9 for African Americans, 12.4 ± 71.9 for Caucasians and 2.5 for other.

The consumption of charbroiled meat during the last trimester continued to be significantly different among racial groups ($p=0.001$). African American women consumed less charbroiled meat during the last trimester in pregnancy compared with Caucasian women and others.

Table 15. Maternal smoking, alcohol intake and other exposures during the third trimester of pregnancy

	African American N=365 (33.1%)* Mean ± s.d. (range)	Caucasian N=722 (65.5%)* Mean ± s.d. (range)	Other N=16 (1.4%)* Mean ± s.d. (range)	Overall N=1,103* Mean ± s.d. (range)
Smoked during the last trimester	88 (58.3%)	213 (56.8%)	4 (66.7%)	305 (57.3%)
Cigarette/day	6.4 ± 7.6 (0.1-40)	9.0 ± 6.9 (0.1-40)	3.3 ± 2.6 (0.3-6)	8.1 ± 7.2 (0.1-40)
Drink alcohol during last trimester	14 (3.8%)	79 (11.5%)	1 (6.3%)	94 (8.2%)
Alcohol (g)/week	5.9 ± 6.9 (0.4-25.6)	12.4 ± 71.9 (0.08-640)	2.5	11.3 ± 65.9 (0.08-640)
Charbroiled meat during last trimester				
Never	120 (32.9%)	182 (25.2%)	3 (18.7%)	305 (27.7%)
< 1x/month	115 (31.5%)	180 (24.9%)	3 (18.7%)	298 (27.0%)
1-4X/month	94 (25.8%)	245 (34.0%)	9 (56.3%)	348 (31.5%)
>4x/month	36 (9.8%)	114 (15.8%)	1 (6.3%)	151 (13.7%)
No response	0	1 (0.1%)	0	1 (0.1%)

* The number of subjects with the third trimester data differs from the number of subjects with baseline demographic data due to missing follow-up questionnaires

3.4 Maternal and newborn phase I/II metabolic enzyme genetic polymorphism allele frequencies

Maternal and newborn allele frequencies of phase I/II metabolic enzyme stratified by ethnicity are summarized in Table 16 and Table 17. The overall population frequencies observed for each racial groups were as we expected based on other existing data [147, 155, 162, 165, 195]. Pairs of maternal/newborn genotypes were verified for Mendelian inherited patterns. In the very rare instances where the genotypes observed were not biologically plausible, the analyses were repeated; no data remained discordant.

Table 16. Maternal allele frequencies of phase I/II metabolic enzyme genetic polymorphisms

	African American N=376 (32.8%)	Caucasian N=754 (65.7%)	Other N=18 (1.5%)	Overall N=1,148
*CYP1A1**				
***1/*1**	186 (49.5%)	572 (75.9%)	9 (50.0%)	767 (66.8%)
*1/*2A, *1/*3	173 (46.0%)	172 (22.8%)	7 (38.9%)	352 (30.7%)
*2A/*2A, *2A/*3, *3/*3	17 (4.5%)	9 (1.3%)	2 (11.1%)	28 (2.5%)
*CYP2E1**				
*1/*1	340 (90.4%)	716 (95.0%)	11 (61.1%)	1067 (92.9%)
*5B/*5B	34 (9.0%)	38 (5.0%)	7 (38.9%)	76 (6.8%)
*5B/*5B	2 (0.5%)	0	0	2 (0.2%)
GSTM1				
Present	242 (64.4%)	359 (47.6%)	13 (72.2%)	614 (53.5%)
Null	134 (35.6%)	395 (52.4%)	5 (27.8%)	534 (46.5%)
GSTT1				
Present	271 (72.1%)	571 (75.7%)	13 (72.2%)	855 (74.5%)
Null	105 (27.9%)	183 (24.3%)	5 (27.8%)	293 (25.5%)
*NAT2**				
Fast	239 (63.6%)	368 (48.9%)	11 (61.1%)	618 (53.9%)
Slow	137 (36.4%)	385 (51.3%)	7 (38.9%)	529 (46.1%)

Note: The number of subjects with allele frequency data differs from the number of subjects with baseline demographic data due to missing genotype data

Table 17. Newborn allele frequencies of phase I/II metabolic enzyme genetic polymorphisms

	African American N=376 (32.8%)	Caucasian N=754 (65.7%)	Other N=18 (1.5%)	Overall N=1,148
*CYP1A1**				
***1/*1**	204 (54.3%)	599 (79.7%)	8 (44.4%)	811 (70.8%)
*1/*2A, *1/*3*	138 (36.7%)	149 (19.8%)	10 (55.6%)	297 (25.9%)
*2A/*2A, *2A/*3, *3/*3*	34 (9.0%)	4 (0.5%)	0	38 (3.3%)
CYP2E1				
*1/*1*	346 (92.0%)	710 (94.2%)	14 (77.8%)	1070 (93.2%)
*1/*5B*	30 (8.0%)	44 (5.8%)	3 (16.7%)	77 (6.7%)
*5B/*5B*	0	0	1 (5.5%)	1 (0.1%)
GSTM1				
Present	246 (65.4%)	347 (46.0%)	12 (66.7%)	605 (52.7%)
Null	130 (34.6%)	407 (54.0%)	6 (33.3%)	543 (47.3%)
*GSTT1**				
Present	276 (73.4%)	606 (80.5%)	12 (66.7%)	894 (77.9%)
Null	100 (26.6%)	147 (19.5%)	6 (33.3%)	253 (22.1%)
*NAT2**				
Fast	236 (62.8%)	352 (46.8%)	10 (55.6%)	598 (52.2%)
Slow	140 (37.2%)	400 (53.2%)	8 (44.4%)	548 (47.8%)

Note: The number of subjects with allele frequency data differs from the number of subjects with baseline demographic data due to missing genotype data

3.5 Classification of low birthweight, preterm delivery and SGA

From among the overall study group, singleton live birth mother/newborn pairs (n=1148) were classified into five groups based on birthweight and gestational weeks as follows: 1) preterm delivery ($<37^{th}$ gestational week) (n=93); 2) low birthweight (<2500 g birthweight) (n=85); 3) preterm and low birthweight ($<37^{th}$ gestational week and <2500 g birthweight) (n=52); 4) SGA ($\leq 10^{th}$ percentile of birthweight at term (≥37 gestational week)); and 5) AGA (n=947) as the referent group. Figure 4 summarizes how these groups are distributed among the total study population.

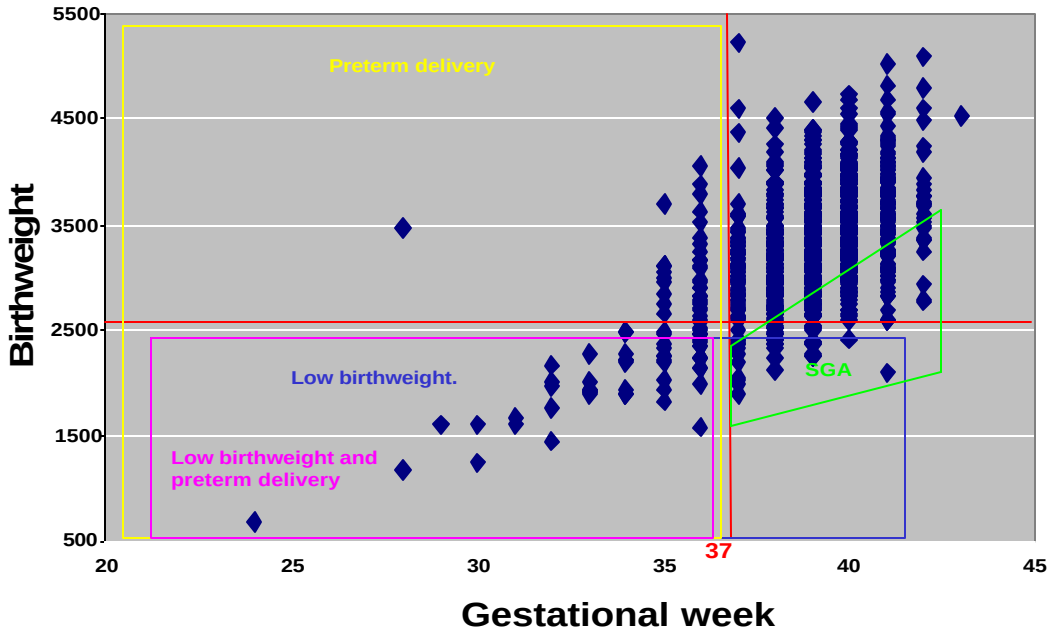

Figure 4. Correlation between birthweight and gestational week in the study population

3.6 Maternal demographic characteristics stratified by low birthweight, preterm delivery and SGA

3.6.1 Association of newborn gender with the adverse reproductive outcomes

A possible gender effect was evaluated for an association with these adverse reproductive outcomes in the fetus. The mean birthweight for female infants was less than that among male infants, but this difference was not statistically significant. Although a greater number of male infants were low birthweight and preterm delivery, this too was not statistically significant.

3.6.2 Low birthweight (<2500g)

Overall, the mean maternal age for low birthweight infants (<2500g) was 23.8 ± 6.1 compared to 25.4 ± 6.3 for AGA mothers, a statistically significant difference (p=0.033). The majority of mothers of low birthweight infants were between 21 and 30 years old (48.8%); a similar age distribution was observed in AGA (49.0%). However, women younger than 15 years old with low birthweight infants were represented at a much higher percentage (3.5%) compared with AGA (0.7%).

In this study, race was a significant risk factor for delivering a low birthweight baby. African American women were more likely to have low birthweight infants compared with Caucasian women (p=0.028). The proportion of low birthweight infant among African Americans was approximately 9.8% while 6.3% for Caucasians.

Family income was also significantly different among the groups of low birthweight infants (p=0.037). The low birthweight infants were more likely to be from the lower family income group (55.9%), defined as <$20,000, while only 40.3% for the AGA group. Moreover, only 16.3% of the low birthweight infant mothers were in the highest income level, while 29.3% women in the AGA group were in this high income level.

Furthermore, maternal education level was significantly different between the low birthweight and AGA groups (p=0.003). Over 70% of women were high school graduates or

less in the low birthweight group compared to 50% of women in the AGA group. Moreover, less than 10% of women in the low birthweight achieved a BS degree or higher compared with over 20% of the women in the AGA.

3.6.3 Preterm delivery (<37th gestational week)

No significant difference in maternal age was observed between the preterm delivery and AGA groups (p=0.408). The mean age was 24.5 ± 5.9 years in preterm delivery while 25.4 ± 6.3 years old in AGA. Approximately half of the women in the both preterm delivery (47.3%) and AGA groups (49.0%) were between age 21 and 30. However, about a three times higher percentages of women were younger than 15 years old in the preterm delivery group (2.1%) compared to the women in the AGA group (0.7%).

There was not a significant difference in ethnicity between the preterm delivery and AGA group (p=0.210). Approximately 9.0% of African American women delivered before 37 gestational weeks compared to 7.6% of Caucasian women.

No significant differences were observed in either family income (p=0.496) or maternal education (p=0.084) between women in the preterm delivery and in AGA groups. Similar to the AGA group, over 20% of the women in the preterm delivery were in the lowest and highest family income groups. For the maternal education, over half of the women in both the preterm delivery and AGA groups were high school graduates or less.

3.6.4 Low birthweight and preterm delivery (<2500g and <37th gestational week)

No significant differences were observed in the following demographic factors; maternal age (p=0.197); ethnicity (p=0.395); and family income (p=0.271) for women in the low birthweight and preterm delivery group compared with women in the AGA group. However, maternal education was significantly different between the low birthweight and preterm delivery and the AGA groups (p=0.029). Women in the low birthweight and preterm delivery group were more likely to be less educated compared with women in the AGA group. Over 70% of the women were high school graduates or less and approximately 7.5% of the women had a BS or higher education in the low birthweight and preterm delivery group. Compared to approximately 50% women had less than a high school graduates and over 20% women had a BS or greater in the AGA group.

3.6.5 Small for gestational age (10th percentile birthweight at full term)

Maternal ages in the SGA and AGA groups were significantly different (p=0.004). Women in the SGA group were more likely to be younger (mean=23.5 ± 5.3 years old) compared with women in the AGA group (mean=25.4 ± 6.3 years old). Approximately half of women in both groups were between age 21 and 30. However, 13.1% women in the SGA group were between age 31-39 and none were age over 40, whereas over 20% of women in the AGA group were 31-39 years old and 2.1% were over 40 years old.

A significant difference was observed in ethnicity between the SGA and AGA groups (p<0.001). African American women had over a two-fold higher risk of having a SGA infant

compared with Caucasian women. Approximately 14.4% of the 376 African American women, had an infant the 10^{th} weight percentile for full term delivery while only 6.9% of the 772 Caucasian women, had SGA infants.

The distribution of family income for the SGA group was skewed to the lower end compared with that for the AGA group and this difference was statistically significant (p=0.001). Nearly 40% of the women in the SGA group were in the <$10,000 family income category whereas only 21% were in the AGA group. On the other hand, approximately a two-fold higher percentage of women in the AGA group (29.3%) were in the highest income category compared with women in the SGA group (15.0%).

Similar to family income, maternal education was also significantly different between these two groups (p=0.014). Women in the SGA group were more likely to have less education compared with women in the AGA group. Over 60% of women in the SGA group were high school graduates or less compared to approximately of 53.3% women in the AGA group.

Table 18. Selected maternal demographic characteristics

	<2500g N=86 Mean ± s.d. (range)	<37th G. W. N=93 Mean ± s.d. (range)	<2500g & <37th G. W. N=53 Mean ± s.d. (range)	SGA N=107 Mean ± s.d. (range)	AGA N=948 Mean ± s.d. (range)	Overall N=1,148 Mean ± s.d. (range)
Maternal age	23.8 ± 6.1 (14-39)	24.5 ± 5.9 (14-39)	24.2 ± 6.2 (14-39)	23.5 ± 5.3 (14-38)	25.4 ± 6.3 (13-45)	25.1 ± 6.2 (13-45)
≤ 15 years old	3 (3.5%)	2 (2.1%)	2 (3.7%)	2 (1.9%)	7 (0.7%)	11 (1.0%)
16-20 years old	27 (31.4%)	29 (31.2%)	16 (30.2%)	32 (29.9%)	257 (27.1%)	318 (27.7%)
21-30 years old	42 (48.8%)	44 (47.3%)	25 (47.2%)	59 (55.1%)	464 (49.0%)	567 (49.4%)
31-39 years old	14 (16.3%)	18 (19.4%)	10 (18.9%)	14 (13.1%)	200 (21.1%)	232 (20.2%)
≥40 years old	0	0	0	0	20 (2.1%)	20 (1.7%)
Ethnicity*						
African American	37 (9.8%)	34 (9.0%)	19 (5.1%)	54 (14.4%)	288 (76.6%)	376
Caucasian	49 (6.3%)	59 (7.6%)	34 (4.3%)	53 (6.9%)	660 (85.4%)	772
Family income:						
<$10,000	27 (31.4%)	20 (21.5%)	11 (20.8%)	40 (37.4%)	206 (21.7%)	266 (23.2%)
$10,000-<$20,000	21 (24.4%)	22 (23.7%)	16 (30.2%)	18 (16.8%)	176 (18.6%)	216 (18.8%)
$20,000-<$35,000	9 (10.5%)	13 (13.9%)	6 (11.2%)	16 (15.0%)	128 (13.5%)	157 (13.7%)
≥$35,000	14 (16.3%)	20 (21.5%)	11 (20.8%)	16 (15.0%)	278 (29.3%)	314 (27.4%)
Don't know	15 (17.4%)	18 (19.4%)	9 (17.0%)	17 (15.8%)	160 (16.9%)	195 (16.9%)
Maternal education *						
<12 grade	18 (20.9%)	21 (22.6%)	12 (22.6%)	28 (26.2%)	175 (18.5%)	224 (19.5%)
High school	44 (51.2%)	36 (38.7%)	26 (49.1%)	44 (41.1%)	330 (34.8%)	410 (35.7%)
diploma	16 (18.6%)	25 (26.9%)	11 (20.8%)	23 (21.5%)	221 (23.3%)	269 (23.4%)
Some collage	8 (9.3%)	11 (11.8%)	4 (7.5%)	12 (11.2%)	222 (23.4%)	245 (21.4%)
BS or greater						

* Indicates statistically significant difference at p= 0.05 between low birthweight, preterm delivery or low birthweight & preterm delivery, SGA and AGA

3.7 Maternal cigarette smoke exposure and alcohol consumption prior to pregnancy stratified by low birthweight, preterm delivery and SGA

3.7.1 Cigarette smoke exposure

Approximately half of women in all groups were current cigarette smokers at baseline (52.9% in low birthweight, 47.3% in preterm delivery, 67.3% in low birthweight and preterm

delivery, 61.7% in SGA and 44.5% in AGA). The mean number of cigarettes smoked per day was 14.3 ± 8.6, 15.3 ± 10.1, 14.2 ± 9.2, 15.0 ± 9.6, and 14.3 ± 8.9, respectively. Over 60% of the women continued to smoke since pregnant (80.0% in low birthweight, 75.0% in preterm delivery, 60.0% in low birthweight and preterm delivery, 72.7% in SGA and 62.7% in AGA). A significant difference was observed in the percentage of women who continued smoking during pregnancy between the low birthweight and AGA groups ($p<0.001$). The average number of cigarettes smoked per day during the pregnancy dramatically dropped substantially in all groups. The mean number of cigarettes smoked per day since pregnant was 9.6 ± 8.6 in low birthweight, 8.0 ± 5.7 in preterm delivery, 8.2 ± 5.2 in low birthweight and preterm delivery, 7.7 ± 8.6 in the SGA group and 7.7 ± 8.6 in the AGA group (Table 19).

Over 35% of women (45.3% of the low birthweight, 39.8% of the preterm delivery, 41.5% of the low birthweight and preterm delivery, 44.9% of the SGA and 36.1% of the AGA groups) were passively exposed to passive cigarette smoke during the early stages of pregnancy. The mean hours of exposure per week for the women in each group were 46.5 ± 36.1, 34.2 ± 28.8, 37.7 ± 29.2, and 36.9 ± 34.7, respectively. A significant difference was observed in the passive cigarette smoke exposure between the low birthweight and AGA groups ($p=0.01$). Approximately 20.9% of women in the low birthweight group reported being passively exposed more than 49 hours per week, while only 10.1% of AGA women were exposed to that level.

3.7.2 Alcohol consumption in early pregnancy

As summarized in Table 19, approximately 70% of women had consumed alcohol prior to pregnancy. The mean alcohol (g) consumed per week was 69.7 ± 79.8 in low birthweight, 64.8 ± 98.9 in preterm delivery, 66.4 ± 63.8 in low birthweight and preterm delivery and 68.5 ± 142.6 in AGA. However, the vast majority of women stopped drinking once pregnant.

Table 19. Maternal cigarette smoke exposure and alcohol consumption prior to pregnancy

	<2500g N=86 Mean ± s.d. (range)	<37th G. W. N=93 Mean ± s.d. (range)	<2500g & <37th G. week N=53 Mean ± s.d. (range)	SGA N=107 Mean ± s.d. (range)	AGA N=948 Mean ± s.d. (range)	Overall N=1,148 Mean ± s.d. (range)
Current smoker (N)	45 (52.9%)	44 (47.3%)	27 (67.3%)	66 (61.7%)	422 (44.5%)	532 (46.3%)
Cigarettes/day prior to pregnancy	14.3 ± 8.6 (2-40)	15.3 ± 10.1 (3-50)	14.2 ± 9.2 (3-40)	15.0 ± 9.6 (2-45)	14.3 ± 8.9 (0.1-50)	14.5 ± 9.1 (0.1-50)
Continue to smoke since pregnant	36 (80.0%)	33 (75.0%)	21 (60.0%)	52 (72.7%)	269 (62.7%)	354 (66.5%)
Cigarettes/day since pregnant	9.6 ± 8.6 (1-40)	8.0 ± 5.7 (1-20)	8.2 ± 5.2 (2-20)	7.7 ± 8.6 (1-40)	7.7 ± 8.6 (0.1-80)	7.6 ± 7.6 (0.1-80)
Passive smoking (N)	39 (45.3%)	37 (39.8%)	22 (41.5%)	48 (44.9%)	342 (36.1%)	427 (37.2%)
Exposed hours/week	46.5 ± 36.1 (1-168)	34.2 ± 28.8 (1-112)	37.7 ± 29.2 (1-108)	45.1 ± 34.5 (2-168)	36.9 ± 34.7 (1-168)	37.5 ± 343
<3.5 h/week	50 (58.1%)	59 (63.4%)	34 (64.2%)	61 (57.0%)	630 (66.5%)	751 (65.2%)
3.5-48 h/week	18 (20.9%)	23 (24.7%)	10 (18.9%)	27 (25.2%)	222 (23.4%)	274 (23.8%)
>49 h/week	18 (20.9%)	11 (11.8%)	9 (17.0%)	19 (17.8%)	96 (10.1%)	127 (11.0%)
Drinker	63 (72.9%)	69 (74.2%)	37 (69.2%)	82	729 (76.9%)	880 (76.7%)
Alcohol(g)/week prior to pregnancy	69.7 ± 79.8 (0.5-347.5)	64.8 ± 98.9 (0.2-642)	66.4 ± 63.8 (0.5-231)	78.1 ± 123.3 (0.5-842)	68.5 ± 142.6 (0.1-1281)	69.1 ± 137.9 (0.41-1281)
Continue to drink since pregnancy	8 (12.7%)	11 (15.9%)	6 (16.2%)	9 (11.0%)	101 (13.9%)	121 (13.8%)

3.7.3 Maternal cigarette smoke exposure and alcohol consumption reported during the third trimester

Overall 35% of women smoked during the third trimester (43.4% in the low birthweight, 35.2% in the preterm, 43.1% in the low birthweight and preterm delivery, and 48.6% in the SGA) and approximately 24.5% in the AGA group. This maternal smoking behavior for each group was significantly different compared with that in the AGA group. The p-values were $p<0.001$, $p=0.025$, $p=0.003$ and $p<0.001$, respectively. The mean number of cigarettes smoked per week in each group was 9.0 ± 9.1, 8.0 ± 8.2, 8.3 ± 8.6, 7.9 ± 6.1 and 8.2 ± 7.3, respectively, and was not significantly different compared with that of the AGA group.

Women in the low birthweight, low birthweight and preterm delivery and SGA groups were more likely to be exposed to cigarette smoke for longer time periods than women in the AGA group. Approximately 40.2% of women in the low birthweight group were exposed longer than 3.5 hours per week during the third trimester compared with women in the AGA group (27.3%). Similarly, there was a higher percentage of women in the SGA group (44.3%) who were exposed more than 3.5 hours per week compared with women in the AGA group. The differences were statistically significant between the AGA and the low birthweight ($p=0.027$), and the SGA groups ($p=0.001$). However, the difference in maternal passive smoke exposure during the third trimester in the preterm delivery and in the low birthweight & preterm delivery groups compared with the AGA group did not reach statistical significance.

The majority of women in all groups did not drink alcohol during the third trimester. For the women who did drink, the mean alcohol (g) consumed per week was 6.7 ± 8.6, 4.0 ± 5.0, 2.2

± 1.2, 8.4 ± 10.3 and 12.1 ± 71.0 in the low birthweight, the preterm delivery, the low birthweight and preterm delivery, the SGA and the AGA groups, respectively.

Table 20. Cigarette smoke exposure and alcohol consumption during the third trimester of pregnancy

Exposure during the third trimester	<2500g N=83 Mean ± s.d. (range)	<37th G. week N=91 Mean ± s.d. (range)	<2500g & <37th G. week N=51 Mean ± s.d. (range)	SGA N=105 Mean ± s.d. (range)	AGA N=907 Mean±s.d. (range)	Overall N=1,103 Mean ± s.d. (range)
Smoked						
Yes	36 (43.4%)	32 (35.2%)	22 (43.1%)	51 (48.6%)	222 (24.5%)	305 (26.6%)
No	47 (56.6%)	59 (64.8%)	29 (56.9%)	54 (51.4%)	685 (75.5%)	798 (72.4%)
Cigarette/day	9.0 ± 9.1 (0.2-30)	8.0 ± 8.2 (0.2-30)	8.3 ± 8.6 (0.2-30)	7.9 ± 6.1 (0.2-20)	8.2 ± 7.3 (0.1-40)	8.1 ± 7.2 (0.1-40)
Passive smoking						
<3.5 hr/week	49 (59.8%)	35 (76.1%)	34 (66.7%)	58 (55.8%)	658 (72.6%)	782 (71.0%)
3.5-49 hr/week	26 (31.7%)	8 (17.5%)	13 (25.5%)	35 (33.7%)	177 (19.5%)	231 (21.0%)
>49 hr/week	7 (8.5%)	3 (6.5%)	4 (7.8%)	11 (10.6%)	71 (7.8%)	88 (8.0%)
Drink alcohol (N)	6 (7.2%)	5 (5.5%)	3 (5.9%)	8 (7.6%)	81 (8.9%)	94 (8.5%)
Alcohol (g)/week	6.7 ± 8.6 (0.64-22.8)	4.0 ± 5.0 (0.8-12.8)	2.2 ± 1.2 (0.8-3.2)	8.4 ± 10.3 (0.3-25.6)	12.1 ± 71.0 (0.08-640)	11.3 ± 65.9 (0.08-640)

* The number of subjects with the last trimester data differs from the number of subjects with baseline demographic data due to missing follow up questionnaires

3.8 Selected phase I/II metabolic enzyme genetic polymorphism allele frequencies

3.8.1 Maternal allele frequencyies of phase I/II metabolic enzyme genetic polymorphisms

Maternal allelic frequencies of selected phase I/II metabolic enzyme genetic polymorphisms classified as low risk or high-risk genotype as previously defined were summarized (Table 21). A significantly higher percentage of women in the low birthweight

group (44.2%) carried at least one *CYP1A1*2A* or *3 variant allele compared with 32.2% of women in the AGA group (p=0.024).

Also, a significantly higher frequency of the *GSTT1* null genotype was observed among women in the low birthweight (p=0.003), the preterm delivery (p=0.004) and the low birthweight and preterm delivery (p=0.002) compared with the frequency observed in the AGA group. However, we did not observe a significantly different *GSTT1* null frequency between low birthweight and AGA groups (p=0.818). In addition, the frequency of the *NAT2** fast acetylator genotype was significantly higher among women in the preterm delivery group (64.5%) as compared with women in the AGA group (p=0.032).

Table 21. Maternal phase I/II metabolic enzyme genetic polymorphism allele frequencies

	<2500g N=86	<37th G. week N=93	<2500g and <37th G. week N=53	SGA N=107	AGA N=948	Overall N=1,148
CYP1A1						
*1/*1	48 (55.8%)	59 (63.4%)	34 (64.1%)	66 (61.7%)	642 (67.8%)	767 (66.8%)
at least one *2A/*3	38 (44.2%)	34 (36.6%)	19 (35.1%)	41 (38.3%)	305 (32.2%)	380 (33.2%)
CYP2E1						
*1/*1	77 (89.5%)	85 (91.4%)	46 (86.8%)	97 (90.7%)	885 (93.4%)	1067 (92.9%)
al least one *5B	9 (10.5%)	8 (8.6%)	7 (13.2%)	10 (9.3%)	63 (6.6%)	81 (7.1%)
GSTM1						
Present	50 (58.1%)	49 (52.7%)	30 (56.6%)	60 (56.1%)	505 (53.3%)	614 (53.5%)
Null	36 (41.9%)	44 (47.3%)	23 (43.4%)	47 (43.9%)	442 (46.7%)	534 (46.5%)
GSTT1						
Present	53 (61.6%)	58 (62.4%)	30 (56.6%)	77 (72.0%)	720 (76.0%)	855 (74.5%)
Null	33 (38.4%)	35 (37.6%)	23 (43.4%)	30 (28.0%)	228 (24.0%)	293 (25.5%)
*NAT2**						
Fast	48 (55.8%)	60 (64.5%)	31 (58.5%)	56 (52.3%)	502 (53.1%)	618 (53.9%)
Slow	38 (44.2%)	33 (35.5%)	22 (41.5%)	51 (47.7%)	445 (46.9%)	529 (46.1%)

* Indicates statistical significant difference at p=0.05 between low birthweight, preterm delivery or low birthweight & preterm delivery, SGA and AGA groups

3.8.2 Newborn allele frequencies of selected phase I/II metabolic enzyme genetic polymorphisms

A significantly higher frequency of the *GSTT1* null genotype among newborns in the preterm delivery group was also observed (p=0.014) compared with that in the AGA referent group. In addition, the differ in *GSTT1* null genotype frequency observed in the low birthweight and preterm delivery group (32.1%) compared to that in the AGA group (21.2%) was very close to significance (p=0.062) (Table 22).

Table 22. Newborn phase I/II metabolic enzyme genetic polymorphism allele frequencies

	<2500g N=86	<37 G. week N=93	<2500g & <37th G. W. N=53	SGA N=107	AGA N=948	Overall N=1,148
CYP1A1						
*1/*1	53 (62.4%)	64 (68.8%)	33 (62.3%)	69 (65.1%)	678 (71.6%)	811 (70.8%)
at least one *2A /*3	32 (37.6%)	29 (31.2%)	20 (37.7%)	37 (34.9%)	269 (28.4%)	335 (29.2%)
CYP2E1						
*1/*1	77 (89.5%)	86 (92.5%)	49 (92.5%)	98 (91.6%)	886 (93.5%)	1074 (93.2%)
al least one *5B	9 (10.5%)	7 (7.5%)	4 (7.5%)	9 (8.4%)	62 (6.5%)	78 (6.8%)
GSTM1						
Present	46 (53.5%)	52 (55.9%)	30 (56.6%)	58 (54.2%)	495 (52.2%)	605 (52.7%)
Null	40 (46.5%)	41 (44.1%)	23 (43.4%)	49 (45.8%)	453 (47.8%)	543 (47.3%)
GSTT1						
Present	62 (72.1%)	63 (67.7%)	36 (67.9%)	85 (79.4%)	746 (78.8%)	894 (77.9%)
Null	24 (27.9%)	30 (32.3%)	17 (32.1%)	22 (20.6%)	201 (21.2%)	253 (22.1%)
*NAT2**						
Fast	51 (60.0%)	51 (54.8%)	32 (60.4%)	60 (56.6%)	487 (51.4%)	598 (52.2%)
Slow	34 (40.0%)	42 (45.2%)	21 (39.6%)	46 (43.4%)	460 (48.6%)	548 (47.8%)

* Indicates statistically significant difference at p= 0.05 between low birthweight, preterm delivery or low birthweight & preterm delivery, SGA and AGA

3.9 Univariate logistic regression analyses

Univariate logistic regression analyses were performed to determine the maternal/newborn lifestyle and genetic factors associated with the following adverse reproductive outcomes: the low birthweight (<2500g), the preterm delivery (<37th gestational week), the low birthweight and preterm delivery (<2500g and <37th week gestational week) and the SGA groups(Table 23 – Table 26).

3.9.1 Univariate logistic regression analysis for low birthweight (<2500g)

As shown in Table 23, African American race was associated with low birthweight (OR=1.74, 95% CI: 1.11-2.73) (p=0.016). Approximately 9.8% of 376 African American women had low birthweight infants while 6.3% of 772 Caucasians had an infant weighting less than 2500 g. Similarly, several other maternal demographic factors were associated with low birthweight including maternal age, lower family income and lower educational level. The ORs were 4.72 (95% CI, 1.18-18.9), 2.62 (95% CI, 1.34-5.11), 2.40 (95% CI, 1.19-4.84), 2.86 (95% CI, 1.21-6.73) and 3.68 (95% CI, 1.70-7.96) for maternal age (≤15 years old), family income (<$10k and $10-<$20k), and maternal education (less than 12 grade and high school graduate), respectively.

Maternal cigarette smoke exposure both active and passive, during the last trimester was clearly associated with low birthweight. The OR was 2.37 (95% CI, 1.49-3.75) and 1.79 (95% CI, 1.12-2.85) for active smoking and passive smoke exposure, respectively. The proportion of

having a low birthweight infant approximately doubled if the mother smoked or was exposed to cigarette smoke during the last trimester.

Mothers with at least one **2A* or **3 CYP1A1* variant allele (p=0.025) or *GSTT1* null (p=0.004) genotypes were at an increased risk of a having low birthweight infant, with the ORs of 1.67 (95% CI, 1.07-2.61) and 1.97 (95% CI, 1.24-3.12) respectively. However, no significant association between any newborn genotype alone and low birthweight was observed.

Table 23. Univariate logistic regression summary for the effects of maternal/newborn demographic, lifestyle and genetic factors on low birthweight (<2,500 g)

Risk factor	N	<2500 g (%)	OR (95% CI)	P value
Maternal demographics				
Ethnicity				
African American*	376	9.8	1.74 (1.11-2.73)	0.016
Caucasian	772	6.3	1.0	
Maternal age (years)				
≤15*	11	27.3	4.72 (1.18-18.9)	0.028
16-20	318	8.5	1.16 (0.70-1.93)	0.56
21-30	567	7.4	1.0	
≥31	252	5.6	0.70 (0.38-1.32)	0.27
Family income				
<$10K*	266	10.2	2.62 (1.34-5.11)	0.005
$10K-<$20K*	216	9.7	2.40 (1.19-4.84)	0.015
$20K-<$35K	157	5.7	1.40 (0.59-3.31)	0.449
≥$35K	314	4.5	1.0	
Don't know	195	7.7	1.86 (0.88-3.96)	0.106
Maternal education				
< 12 grade*	224	8.0	2.86 (1.21-6.73)	0.016
High school diploma*	410	10.7	3.68 (1.70-7.97)	0.001
Some collage	269	5.9	2.01 (0.84-4.79)	0.116
BS or greater	245	3.3	1.0	
Exposure in early pregnancy				
Smoking				
No	616	6.7	1.0	
Yes	532	8.5	1.37 (0.88-2.16)	0.17
Passive				
No	750	6.7	1.0	
Yes	398	9.0	1.43 (0.91-2.24)	0.12
Alcohol use				
No	169	6.5	1.0	
Yes	979	7.7	0.74 (0.33-1.69)	0.48
Exposure during 3rd trimester				
Smoking				
No	798	5.9	1.0	
Yes*	305	11.8	2.37 (1.49-3.75)	<0.001
Passive				
No	782	6.3	1.0	
Yes*	319	10.3	1.79 (1.12-2.85)	0.014
Alcohol use				
No	1002	7.7	1.0	
Yes	99	6.1	0.74 (0.33-1.69)	0.48
Risk group				
Low risk	305	7.5	1.0	
Medium risk	721	7.2	0.99 (0.59-1.65)	0.97
High risk	77	10.4	1.47 (0.63-3.43)	0.38

Table 23 cont.

Maternal genotype				
CYP1A1				
**1/*1*	688	7.0	1.0	
at least one copy of **2A* or **3**	342	11.1	1.67 (1.07-2.61)	0.025
CYP2E1				
**1/*1*	959	8.0	1.0	
at least one copy of **5B*	72	12.5	1.64 (0.78-3.42)	0.19
GSTM1				
Present	552	9.1	1.0	
Null	479	7.5	0.82 (0.52-1.28)	0.37
*GSTT1**				
Present	771	6.9	1.0	
Null*	260	12.7	1.97 (1.24-3.12)	0.004
*NAT2**				
Fast	548	8.8	1.0	
Slow	482	7.9	0.89 (0.57-1.39)	0.61
Newborn genotype				
CYP1A1				
**1/*1*	729	7.3	1.0	
at least one copy of **2A* or **3*	300	10.7	1.52 (0.96-2.41)	0.074
CYP2E1				
**1/*1*	960	8.0	1.0	
at least one copy of **5B*	71	12.7	1.66 (0.80-3.48)	0.175
GSTM1				
Present	540	8.5	1.0	
Null	491	8.2	0.95 (0.61-1.48)	0.83
GSTT1				
Present	806	7.7	1.0	
Null	224	10.7	1.44 (0.88-2.37)	0.15
*NAT2**				
Fast	535	9.5	1.0	
Slow	494	6.9	0.70 (0.44-1.10)	0.12

* Indicates statistically significant difference at p= 0.05

3.9.2 Univariate logistic regression analysis for preterm delivery (<37th week gestation)

Maternal education level was associated with preterm delivery with an OR of 2.42 (95% CI, 1.14-5.16), 2.19 (95% CI, 1.09-4.40) and 2.28 (95% CI, 1.10-4.75) for women with < 12 grade, those who were high school graduates, and those with some college degree, respectively compared to the AGA group.

Maternal cigarette smoking during the last trimester was significantly associated with preterm delivery (p=0.026) with an OR of 1.68 (95% CI, 1.06-2.65). However, no association between passive cigarette smoke exposure during the last trimester and preterm delivery (p=0.98) was observed.

Not only maternal (p=0.004) but also newborn (p=0.015) *GSTT1* null genotype was clearly associated with preterm delivery. The OR was 1.91 (95% CI, 1.22-2.98) and 1.77 (95% CI, 1.12-2.81) for maternal and newborn *GSTT1* null genotype, respectively. In addition, an inverse association was observed between maternal *NAT2** slow acetylator genotype and preterm delivery with an OR of 0.62 (95% CI, 0.40-0.97).

Table 24. Univariate logistic regression summary for the effects of maternal/newborn demographic, lifestyle and genetic factors on preterm delivery (<37th week gestation)

Risk factor	N	<37 G. week (%)	OR (95% CI)	P value
Maternal demographics				
Ethnicity				
African American	376	9.0	1.33 (0.85-2.07)	0.21
Caucasian	772	7.6	1.0	
Maternal age (years)				
≤15	11	18.2	3.01 (0.61-14.9)	0.18
16-20	318	9.1	1.19 (0.73-1.95)	0.49
21-30	567	7.8	1.0	
≥31	252	7.1	0.86 (0.49-1.53)	0.62
Family income				
<$10K	266	7.5	1.36 (0.71-2.59)	0.36
$10K-<$20K	216	10.2	1.75 (0.93-3.31)	0.082
$20K-<$35K	157	8.3	1.41 (0.68-2.93)	0.35
≥$35K	314	6.4	1.0	
Don't know	195	9.2	1.56 (0.80-3.04)	0.19
Maternal education				
< 12 grade	224	9.4	2.42 (1.14-5.16)	0.022
High school diploma	410	8.8	2.19 (1.09-4.40)	0.027
Some collage	269	9.3	2.28 (1.10-4.75)	0.027
BS or greater	245	4.5	1.0	
Exposure in early pregnancy				
Smoking				
No	616	8.0	1.0	
Yes	532	8.3	1.12 (0.73-1.71)	0.61
Passive				
No	750	7.9	1.0	
Yes	398	8.5	1.14 (0.73-1.78)	0.56
Alcohol use				
No	169	7.1	1.0	
Yes	979	8.3	1.18 (0.63-2.23)	0.60
Exposure during 3rd trimester				
Smoking				
No	798	7.4	1.0	
Yes*	305	10.5	1.68 (1.06-2.65)	0.026
Passive				
No	782	8.4	1.0	
Yes	319	7.8	1.01 (0.62-1.63)	0.98
Alcohol use				
No	1002	8.6	1.0	
Yes	99	5.1	0.56 (0.22-1.41)	0.22
Risk group				
Low risk	305	8.2	1.0	
Medium risk	721	8.5	1.07 (0.66-1.74)	0.79
High risk	77	6.5	0.84 (0.31-2.29)	0.74

Table 24 cont.

Maternal genotype				
CYP1A1				
**1/*1*	699	8.4	1.0	
at least one copy of **2A* or **3*	338	10.1	1.21 (0.77-1.89)	0.39
CYP2E1				
**1/*1*	967	8.1	1.0	
at least one copy of **5B*	71	11.3	1.32 (0.61-2.84)	0.48
GSTM1				
Present	551	8.9	1.0	
Null	487	9.0	1.02 (0.66-1.56)	0.94
GSTT1				
Present	776	7.5	1.0	
Null*	262	13.4	1.91 (1.22-2.98)	0.004
*NAT2**				
Fast	560	10.7	1.0	
Slow*	477	6.9	0.62 (0.40-0.97)	0.034
Newborn genotype				
CYP1A1				
**1/*1*	740	8.7	1.0	
at least one copy of **2A* or **3*	297	9.8	1.14 (0.72-1.81)	0.57
CYP2E1				
**1/*1*	969	8.9	1.0	
at least one copy of **5B*	69	10.1	1.16 (0.51-2.61)	0.72
GSTM1				
Present	546	9.5	1.0	
Null	492	8.3	0.86 (0.56-1.33)	0.50
GSTT1				
Present	807	7.8	1.0	
Null*	230	13.0	1.77 (1.12-2.81)	0.015
*NAT2**				
Fast	535	9.5	1.0	
Slow	502	8.4	0.87 (0.56-1.33)	0.51

* Indicates statistically significant difference at p= 0.05

3.9.3 Univariate logistic regression analysis for low birthweight and preterm delivery (<2500g and <37th week gestation)

A similar set of risk factors was observed for the low birthweight and preterm delivery group as seen for the low birthweight group. African American women had a significantly higher risk of having a low birthweight infant, delivered at less than 37 gestational weeks (p=0.032) with an OR of 1.29 (95% CI, 0.72-2.30). Moreover, maternal age (younger than 15 years old) was strongly associated with preterm delivery (OR=5.29, 95% CI, 1.04-26.8). Lower family income and lower maternal education level were also risk factors for both low birthweight and preterm delivery with an OR of 2.32 (95% CI, 1.05-5.12), 3.81 (95% CI, 1.21-12.02) and 4.35 (95% CI, 1.50-12.64) for family income $10K-<$20K, less than 12 grade maternal education and high school graduate, respectively.

Active maternal cigarette smoking during the third trimester was clearly associated with low birthweight and preterm delivery (p=0.004) with an OR of 2.34 (95% CI, 1.32-4.16). The proportion of infants with low birthweight and preterm delivery was approximately doubled (7.2%) if the mother smoked in late pregnancy.

Maternal *GSTT1* null genotype significantly increased the risk of low birthweight and preterm delivery (p=0.002); the OR was 2.42 (95% CI, 1.38-4.26). Newborn *GSTT1* null genotype also increased the risk (OR=1.76, 95% CI, 0.97-3.19), however, it was not statistically significant (p=0.065).

Table 25. Univariate logistic regression summary for the effects of maternal/newborn demographic, lifestyle and genetic factors on low birthweight and preterm delivery (<2500 g and <37th week gestation)

Risk factor	N	<2500g &<37th G.week (%)	OR (95% CI)	P value
Maternal demographics				
Ethnicity				
African American*	376	5.1	1.29 (0.72-2.30)	0.032
Caucasian	772	4.4	1.0	
Maternal age (years)				
≤15*	11	18.2	5.29 (1.04-26.8)	0.044
16-20	318	5.0	1.16 (0.61-2.21)	0.66
21-30	567	4.4	1.0	
≥31	252	3.4	0.85 (0.40-1.79)	0.66
Family income				
<$10K	266	4.1	1.36 (0.58-3.19)	0.49
$10K-<$20K*	216	7.4	2.32 (1.05-5.12)	0.037
$20K-<$35K	157	3.8	1.18 (0.43-3.27)	0.74
≥$35K	314	3.5	1.0	
Don't know	195	4.6	1.42 (0.58-3.50)	0.45
Maternal education				
< 12 grade*	224	5.4	3.81 (1.21-12.02)	0.022
High school diploma*	410	6.3	4.35 (1.50-12.64)	0.007
Some collage	269	4.1	2.76 (0.87-8.81)	0.086
BS or greater	245	1.6	1.0	
Exposure in early pregnancy				
Smoking				
No	616	4.2	1.0	
Yes	532	5.1	1.29 (0.74-2.25)	0.36
Passive				
No	750	4.5	1.0	
Yes	398	4.8	1.11 (0.62-1.97)	0.73
Alcohol use				
No	169	4.1	1.0	
Yes	979	4.7	1.15 (0.51-2.60)	0.73
Exposure during 3rd trimester				
Smoking				
No	798	3.6	1.0	
Yes*	305	7.2	2.34 (1.32-4.16)	0.004
Passive				
No	782	4.3	1.0	
Yes	319	5.3	1.33 (0.73-2.42)	0.35
Alcohol use				
No	1002	4.8	1.0	
Yes	99	3.0	0.60 (0.18-1.95)	0.40
Risk group				
Low risk	305	4.9	1.0	

Table 25 cont.

Medium risk	721	4.4	0.94 (0.50-1.76)	0.84
High risk	77	5.2	1.12 (0.36-3.50)	0.84
Maternal genotype				
CYP1A1				
**1/*1*	674	5.0	1.0	
at least one copy of **2A* or **3*	323	5.9	1.17 (0.66-2.10)	0.58
CYP2E1				
**1/*1*	928	5.0	1.0	
at least one copy of **5B*	70	10.0	2.13 (0.94-4.91)	0.076
GSTM1				
Present	532	5.6	1.0	
Null	466	4.9	0.87 (0.50-1.52)	0.62
GSTT1				
Present	748	4.0	1.0	
Null*	250	9.2	2.42 (1.38-4.26)	0.002
*NAT2**				
Fast	531	5.8	1.0	
Slow	466	4.7	0.80 (0.46-1.40)	0.434
Newborn genotype				
CYP1A1				
**1/*1*	709	4.7	1.0	
at least one copy of **2A* or **3*	288	6.9	1.53 (0.86-2.71)	0.15
CYP2E1				
**1/*1*	932	5.3	1.0	
at least one copy of **5B*	66	6.1	1.16 (0.41-3.33)	0.78
GSTM1				
Present	524	5.7	1.0	
Null	474	4.9	0.84 (0.48-1.47)	0.54
GSTT1				
Present	780	4.6	1.0	
Null*	217	7.8	1.76 (0.97-3.19)	0.065
*NAT2**				
Fast	516	6.2	1.0	
Slow	481	4.4	0.69 (0.39-1.21)	0.20

* Indicates statistically significant difference at p= 0.05

3.9.4 Univariate logistic regression analysis for SGA at term (≥ 37th week gestation)

From Table 26, it can be seen that the ethnicity was clearly associated with SGA ($p<0.001$) with an OR of 2.33 (95% CI, 1.56-3.50). Approximately 14.4% of African American women had an infant with SGA at full term, whereas this was observed in only 6.9% of Caucasians. Women with lower family income and lower education level were also at a significantly increased risk for SGA infants with an OR of 3.37 (95% CI, 1.84-6.19), 2.17 (95% CI, 1.05-4.48), 2.96 (95% CI, 1.46-5.99), and 2.47 (95% CI, 1.27-4.78) for <$10K, and $20K-<$35K family income and maternal education (< 12 grade, and high school graduate), respectively. Also women over 31 years old had a reduced risk of having a SGA infant (OR=0.50 95% CI, 0.27-0.92)

Maternal cigarette smoke exposure during pregnancy was associated with SGA with an OR of 2.01 (95% CI, 1.33-3.02) for early pregnancy smoking and 2.91 (95% CI, 1.93-1.74) for smoking during the third trimester. Passive cigarette smoke exposure in late pregnancy also increased the risk of SGA (OR=2.10 95% CI, 1.39-3.18). Moreover, the third trimester maternal exposure risk group was significantly associated with SGA in a dose-responsive fashion (OR=2.22, 95% CI, 1.02-4.86 in high risk).

No associations of the selected phase I/II metabolic enzyme genetic polymorphisms in mothers or newborns with SGA was observed.

Table 26. Univariate logistic regression summary for the effects of maternal/newborn demographic, lifestyle and genetic factors on SGA at term

Risk factor	N	SGA (%)	OR (95% CI)	P value
Maternal demographics				
Ethnicity				
African American*	376	14.4	2.33 (1.56-3.50)	<0.001
Caucasian	772	6.9	1.0	
Maternal age (years)				
≤15	11	18.2	2.25 (0.46-11.07)	0.32
16-20	318	10.1	0.98 (0.62-1.55)	0.93
21-30	567	10.4	1.0	1.0
≥31*	252	5.6	0.50 (0.27-0.92)	0.025
Family income				
<$10K*	266	15.0	3.37 (1.84-6.19)	<0.001
$10K-<$20K	216	8.3	1.78 (0.88-3.58)	0.12
$20K-<$35K*	157	10.2	2.17 (1.05-4.48)	0.036
≥$35K	314	5.1	1.0	
Don't know	195	8.7	1.85 (0.91-3.75)	0.09
Maternal education				
< 12 grade*	224	12.5	2.96 (1.46-5.99)	0.003
High school diploma*	410	10.7	2.47 (1.27-4.78)	0.007
Some collage*	269	8.6	1.93 (0.93-3.96)	0.075
BS or greater	245	4.9	1.0	
Exposure in early pregnancy				
Smoking				
No	616	6.7	1.0	
Yes*	532	12.4	2.01 (1.33-3.02)	0.001
Passive				
No	750	8.1	1.0	
Yes	398	11.6	1.49 (1.00-2.24)	0.053
Alcohol use				
No	169	9.5	1.0	
Yes	979	9.3	0.99 (0.57-1.74)	0.98
Exposure during 3rd trimester				
Smoking				
No	798	6.8	1.0	
Yes*	305	16.7	2.91 (1.93-4.40)	<0.001
Passive				
No	782	7.4	1.0	
Yes*	319	14.4	2.10 (1.39-3.18)	<0.001
Alcohol use				
No	1002	9.5	1.0	
Yes	99	9.1	1.20 (0.88-1.63)	0.25
Risk group				
Low risk	305	6.9	1.0	
Medium risk	721	10.1	1.53 (0.92-2.55)	0.098
High risk*	77	14.3	2.22 (1.02-4.86)	0.045

Table 26 cont.

Maternal genotype				
CYP1A1				
**1/*1*	708	9.3	1.0	
at least one copy of **2A* or **3*	346	11.9	1.30 (0.87-1.98)	0.20
CYP2E1				
**1/*1*	982	9.9	1.0	
at least one copy of **5B*	73	13.7	1.45 (0.72-2.91)	0.30
GSTM1				
Present	565	10.6	1.0	
Null	490	9.6	0.89 (0.60-1.33)	0.58
GSTT1				
Present	797	9.7	1.0	
Null	258	11.6	1.23 (0.79-1.92)	0.36
*NAT2**				
Fast	558	10.0	1.0	
Slow	496	10.3	1.02 (0.69-1.53)	0.90
Newborn genotype				
CYP1A1				
**1/*1*	747	9.2	1.0	
at least one copy of **2A* or **3*	306	12.1	1.35 (0.88-2.06)	0.16
CYP2E1				
**1/*1*	984	10.0	1.0	
at least one copy of **5B*	71	12.7	1.31 (0.63-2.72)	0.47
GSTM1				
Present	553	10.5	1.0	
Null	502	9.8	0.92 (0.62-1.48)	0.70
GSTT1				
Present	831	10.2	1.0	
Null	223	9.9	0.96 (0.59-1.57)	0.87
*NAT2**				
Fast	547	11.0	1.0	
Slow	506	9.1	0.81 (0.54-2.10)	0.31

* Indicates statistically significant difference at p= 0.05

3.10 Multiple logistic regression analysis

Multiple logistic regression analysis was performed to determine maternal/newborn lifestyle and/or genetic risk factors for adverse reproductive outcomes: low birthweight, preterm delivery, low birthweight and preterm delivery and SGA at term. Moreover, the analysis was performed to evaluate the modulation of the percentage of adverse reproductive outcomes by combinations of maternal/newborn phase I/II metabolic enzyme genetic polymorphisms. Maternal demographic and lifestyle risk factors, which were significantly associated with the adverse reproductive outcomes in univariate analyses, were included in the multiple logistic regression model for their independent significance. If the factor did not reach significance, it was removed from the model.

Once the risk factors which were associated with these adverse reproductive outcomes were identified by multiple logistic regression analyses, the combined association of these risk factors was evaluated by creating subgroups. These subgroups contained different combinations of these risk factors. The observed and calculated expected proportions of each subgroup for a specific adverse reproductive outcome were determined by the goodness-of-fit test.

3.10.1 Multiple logistic regression analysis for low birthweight (<2500g)

As shown in Table 27, ethnicity, maternal smoking during the 3rd trimester and maternal and newborn *GSTT1* genotype were significantly associated with low infant birthweight. The combinations of these three risk factors clearly influenced the proportion of low birthweight newborns. Among non-smoking Caucasian women, maternal or newborn *GSTT1* genotype alone did not confer a significant risk of low birthweight. However, in the presence of maternal

smoking during the 3rd trimester, the highest observed proportion of low birthweight infants (0.2941) was found when mothers were African American and carried the *GSTT1* null genotype with OR of 7.81 (p= 0.001).

Table 27. Multivariate logistic regression model for the effects of maternal ethnicity, smoking in the 3rd trimester, and maternal/newborn *GSTT1* genotype on low birthweight (<2500 g)

Ethnicity	Smoking during 3rd trimester	*GSTT1* genotype	N	Observed proportion	Expected proportion	OR (95% CI)	p-value
		Maternal					
Caucasian	No	Present	375	0.0507	0.0411	1.0	
		Null	115	0.0435	0.0827	0.85 (0.31-2.33)	0.76
African American	No	Present	180	0.0722	0.0744	1.46 (0.70-3.02)	0.31
		Null	60	0.1667	0.1448	3.75 (1.65-8.52)	0.002
Caucasian	Yes	Present	130	0.0769	0.0940	1.56 (0.71-3.45)	0.27
		Null	55	0.2364	0.1793	5.80 (2.67-12.58)	<0.001
African American	Yes	Present	55	0.1455	0.1631	3.19 (1.32-7.69)	0.01
		Null	17	0.2941	0.2908	7.81 (2.49-24.43)	<0.001
		Newborn					
Caucasian	No	Present	392	0.0408	0.0479	1.0	
		Null	97	0.0825	0.0668	2.11(0.88-5.09)	0.10
African American	No	Present	180	0.0833	0.0829	2.14 (1.03-4.42)	0.041
		Null	60	0.1333	0.1140	3.62 (1.47-8.86)	0.005
Caucasian	Yes	Present	151	0.1192	0.1103	3.18 (1.58-6.42)	0.001
		Null	34	0.1471	0.1501	4.05 (1.39-11.84)	0.011
African American	Yes	Present	53	0.2075	0.1823	6.15 (2.68-14.13)	<0.001
		Null	19	0.1053	0.2410	2.76 (0.59-13.00)	0.198

After the identification of significant associated factors for low birthweight which were maternal ethnicity, maternal cigarette smoking during the 3rd trimester and maternal/newborn *GSTT1* genotype by the multivariable logistic regression, the combined association of these factors was evaluated by creating 8 subgroups. Each subgroup contained a different combination of the risk factors. As shown in Table 27, the observed proportion and expected proportion

values of having a low birthweight infant were calculated. The observed proportion value was calculated by dividing the total number of subjects by the number of the low birthweight subjects in a specific risk factor subgroup. For example, of the 375 total subjects who were non-smoking Caucasians with the *GSTT1* present maternal genotype, 19 delivered a low birthweight infant, giving the observed proportion value of 0.0507. Based on the model predicted ORs, the expected proportion value was calculated. The goodness of fit test was applied to the estimated model to evaluate the quantitative relationship between the observed proportions and those predicted by the model.

Figure 5 presents the combined association of maternal ethnicity, cigarette smoking during the 3rd trimester and maternal or newborn *GSTT1* genotype with observed and modeled proportions of low birthweight infants. The histogram represents the modeled low birthweight proportion by logistic regression and the circles indicate the observed low birthweight proportion in the study. A higher proportion of low birthweight infants was observed if the mother was *GSTT1* null genotype along with African American ethnicity and/or cigarette smoking in the third trimester.

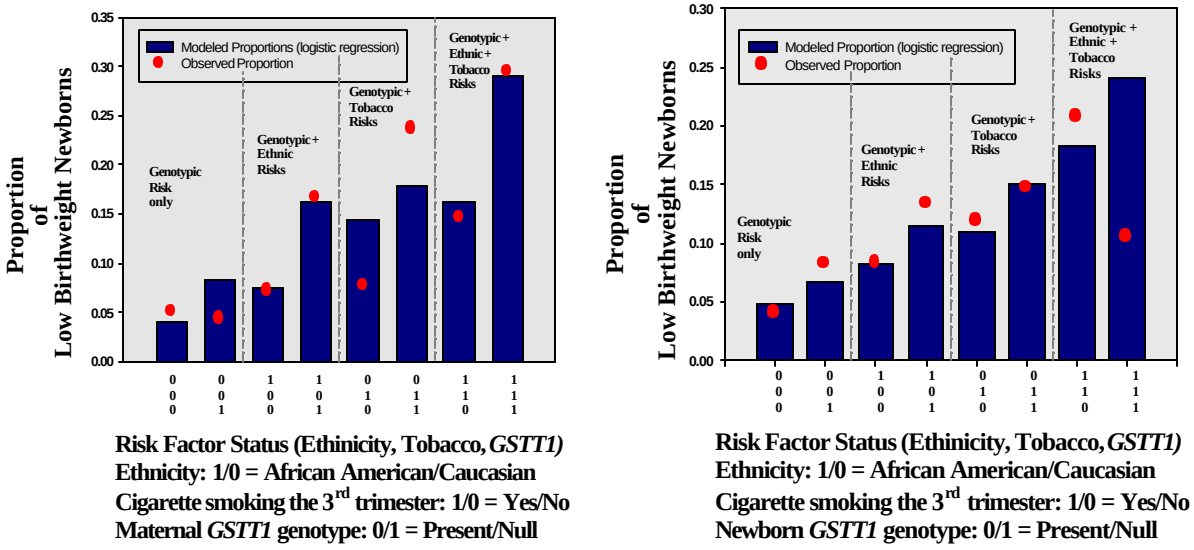

Figure 5. Combined association of maternal ethnicity, cigarette smoking during the 3rd trimester and maternal or newborn *GSTT1* genotype with the proportion of having low birthweight infants

Table 28 presents the combined association of maternal ethnicity, cigarette smoking during the 3rd trimester along with combination of maternal/newborn *GSTT1* genotype with the observed proportion of low birthweight infants.

Among non-smoking Caucasian and African American women, the combination of null *GSTT1* genotype in both maternal/newborn pairs alone did not confer a significant elevation in risk of low birthweight. In the presence of maternal smoking but absence of the null *GSTT1* genotype, a significant elevation of low birthweight births was observed in both Caucasian and African American women. The highest proportion of low birthweight infants was observed

among the African Americans where mother were *GSTT1* null genotype, and in the presence of cigarette smoking exposure (OR=10.82, observed proportion=0.3333, p=0.002).

Table 28. Multivariate logistic regression model for combined risk of low birthweight conferred by maternal/newborn *GSTT1* genotypes, ethnicity and maternal smoking during the 3rd trimester

Ethnicity	Smoking during 3rd trimester	Maternal *GSTT1* genotype	Newborn *GSTT1* genotype	N	Observed proportion	Expected proportion	OR (95% CI)	P-value
Caucasian	No	Present	Present	317	0.0442	0.0426	1.0	
			Null	57	0.0877	0.0348	2.08 (0.72-6.02)	0.176
		Null	Present	75	0.0267	0.0677	0.59 (0.13-2.67)	0.496
			Null	40	0.0750	0.1094	1.75 (0.48-6.39)	0.394
African American	No	Present	Present	145	0.0759	0.0763	1.77 (0.79-4.02)	0.167
			Null	35	0.0571	0.0627	1.31 (0.29-6.02)	0.727
		Null	Present	35	0.1143	0.1188	2.79 (0.87-9.01)	0.086
			Null	25	0.2400	0.1857	6.83 (2.36-19.78)	<0.001
Caucasian	Yes	Present	Present	114	0.0877	0.0974	2.08 (0.90-4.83)	0.088
			Null	16	0.00	0.0804		
		Null	Present	37	0.2162	0.1498	5.97 (2.31-15.41)	<0.001
			Null	18	0.2778	0.2295	8.32 (2.60-26.61)	<0.001
African American	Yes	Present	Present	44	0.1818	0.1668	4.81 (1.89-12.25)	0.001
			Null	11	0.00	0.1396		
		Null	Present	9	0.3333	0.2464	10.82 (2.45-47.82)	0.002
			Null	8	0.2500	0.3561	7.21 (1.33-39.00)	0.022

Figure 6 indicates combined risk status: ethnicity, cigarette smoking during the 3rd trimester and combinations of maternal/newborn *GSTT1* genotype with modeled and observed proportion of low birthweight births. A smoking dose-response association was observed in each of the risk status groups.

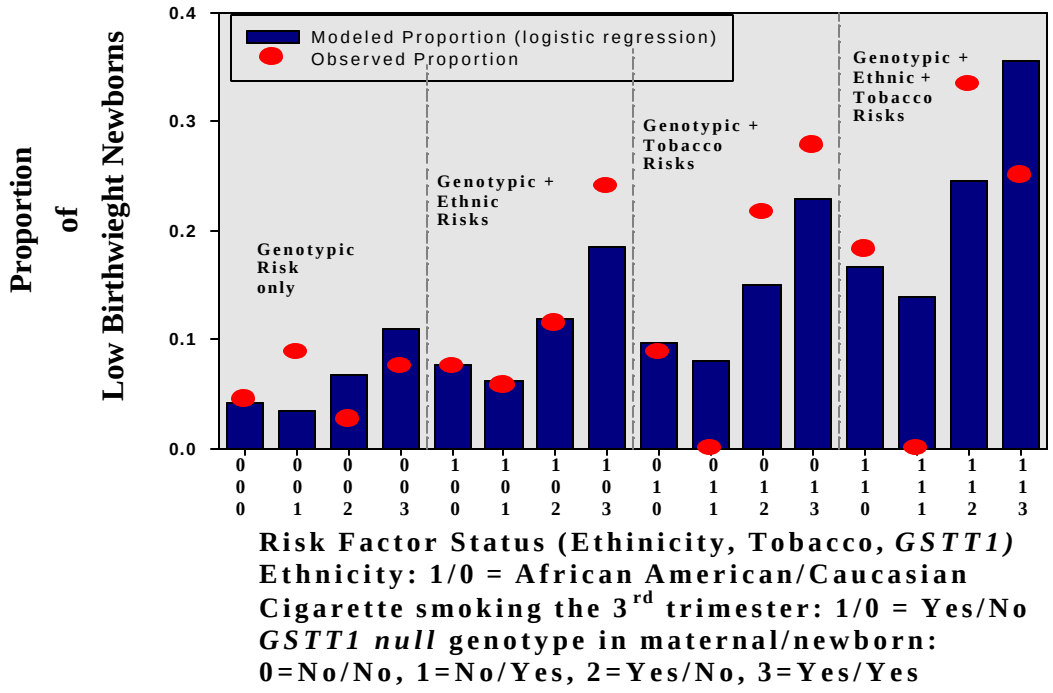

Figure 6. Combined association of maternal ethnicity, cigarette smoking during the 3rd trimester and combination of maternal/newborn *GSTT1* genotypes with proportion of low birthweight newborns

3.10.2 Multiple logistic regression analysis for preterm delivery (<37th week gestation)

As shown in Table 29, a combined risk factor association was observed for maternal cigarette smoke during the 3rd trimester and maternal or newborn *GSTT1* genotype with infant preterm delivery. Among non-smoking women, *GSTT1* genotype alone did not confer a significant risk of shortened gestational weeks. However, the combination of maternal smoking during the third trimester and maternal or newborn *GSTT1* null genotype significantly elevated

the proportion of preterm infants with ORs of 3.87 and 2.48 for maternal or newborn *GSTT1* null genotype, respectively (p<0.001, p=0.030).

Moreover, the combined risk factors of maternal smoking and *NAT2** genotype were also clearly associated with preterm delivery independent of *GSTT1* genotype. Maternal fast acetylators had a significantly higher proportion of preterm delivery infants (OR=2.24 and 2.80) in both non-smoking and smoking women, respectively (p=0.007, 0.004).

Table 29. Multivariate logistic regression model for maternal cigarette smoking in the 3rd trimester and maternal or newborn *GSTT1* and *NAT2** genotypes on preterm delivery

Genotype	Smoking during 3rd trimester	N	Observed proportion	OR (95% CI)	P-value
GSTT1 (Maternal)					
Present	No	563	0.0710	1.0	
	Yes	183	0.0874	1.55 (0.87-2.76)	0.133
Null	No	179	0.1061	1.25 (0.68-2.29)	0.466
	Yes	70	0.2286	3.87 (2.03-7.38)	<0.001
GSTT1 (Newborn)					
Present	No	579	0.0656	1.0	
	Yes	199	0.1206	2.12 (1.21-3.73)	0.009
Null	No	162	0.1296	1.95 (1.14-3.35)	0.015
	Yes	54	0.1481	2.48 (1.09-5.62)	0.030
*NAT2** (Maternal)					
Fast	No	400	0.1050	2.24 (1.25-4.02)	0.007
	Yes	141	0.1261	2.80 (1.40-5.60)	0.004
Slow	No	342	0.0497	1.0	
	Yes	111	0.1277	2.76 (1.31-5.80)	0.007

A combination of maternal/newborn *GSTT1* genotypes further modulated the proportion of preterm delivery infants (Table 30). Among non-smoking women, maternal or newborn *GSTT1* null genotype did not confer a significant adverse effect on preterm delivery. However,

if both mother and infant were of *GSTT1* null genotype, approximately a three fold increased risk of preterm delivery was observed (OR of 3.17 and p=0.001). In the presence of maternal cigarette smoking, during the last trimester, the preterm delivery proportion dramatically increased and the highest proportion was observed among the group of both maternal and newborn *GSTT1* null genotype (OR=4.31, p=0.004).

Table 30. Multivariate logistic regression model for combined risk of preterm delivery (<37th week gestation) conferred by maternal/newborn *GSTT1* genotypes and maternal smoking in the 3rd trimester

Smoking during 3rd trimester	Maternal *GSTT1* genotype	Newborn *GSTT1* genotype	N	Observed proportion of preterm delivery	Expected proportion of preterm delivery	OR (95% CI)	P-value
No	Present	Present	469	0.0682	0.0644	1.0	
		Null	93	0.0860	0.0720	1.29 (0.57-2.89)	0.54
	Null	Present	110	0.0545	0.0886	0.79 (0.32-1.93)	0.60
		Null	69	0.1884	0.1791	3.17 (1.57-6.40)	0.001
Yes	Present	Present	154	0.0909	0.1026	1.37 (0.71-2.63)	0.35
		Null	29	0.0690	0.1140	1.01 (0.23-4.45)	0.99
	Null	Present	45	0.2222	0.1390	3.90 (1.77-8.59)	0.001
		Null	25	0.2400	0.2658	4.31 (1.61-11.55)	0.004

Figure 7 clearly demonstrates the combined association of maternal smoking during the 3rd trimester and combination of maternal/newborn *GSTT1* genotype with preterm delivery.

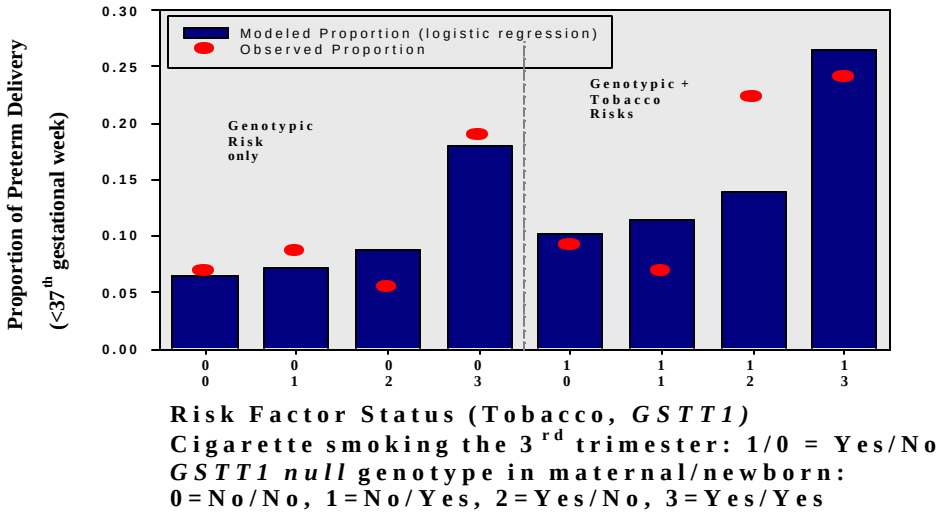

Figure 7. Combined association of exposure factors: maternal cigarette smoking during the 3rd trimester and combination of maternal/newborn *GSTT1* genotype with proportion of preterm delivery infants

3.10.3 Multiple logistic regression analysis for low birthweight and preterm delivery (<2500g and <37th week gestation)

A similar set of risk factors was observed in low birthweight and preterm delivery infants (<2500 g and <37th week gestation) analysis. The highest proportion of low birthweight and preterm delivery (0.2059) was found among smoking women with GSTT1 null genotype with an OR of 6.78 p<0.001 (Table 31). The combination of maternal cigarette smoking during the 3rd trimester and newborn GSTT1 null genotype clearly elevated the proportion of low birthweight infants at less than 37th weeks of gestation. Among non-smoking women, the proportion increased from 0.0322 (referent group) to 0.0885 (OR=2.92, p=0.002) for presence and absence

of newborn GSTT1 genotype, respectively. Similarly, among smoking women, the proportion elevated from 0.0724 (OR=2.34, p=0.031) to 0.0980 (OR=3.27, p=0.025) for presence and absence of newborn *GSTT1* genotype, respectively.

Table 31. Multivariate logistic regression model for the effects of maternal smoking in the 3rd trimester and maternal and/or newborn *GSTT1* genotype, on low birthweight and preterm delivery (<2500g and <37th week gestation)

GSTT1 Genotype	Smoking during 3rd trimester	N	Observed proportion of <2500g & <37G.W.	OR (95% CI)	P-value
Maternal:					
Present	No	543	0.0368	1.0	
	Yes	169	0.0533	1.47 (0.66-3.29)	0.35
Null	No	175	0.0457	1.25 (0.54-2.90)	0.60
	Yes	68	0.2059	6.78 (3.24-14.18)	<0.001
Newborn:					
Present	No	559	0.0322	1.0	
	Yes	152	0.0724	2.34 (1.08-5.08)	0.031
Null	No	192	0.0885	2.92 (1.47-5.79)	0.002
	Yes	51	0.0980	3.27 (1.16-9.20)	0.025

As shown in Table 32 and Figure 7, the combination of maternal/newborn *GSTT1* genotype modulated the proportion of adverse effects on birthweight and gestational period in both the presence and absence of maternal cigarette smoking exposure during the 3rd trimester. The highest observed proportion (0.2083) was found among the group of both mother and newborn *GSTT1* null genotype in the presence of cigarette smoking exposure (OR=7.19, p<0.001). Moreover, a dose-response association of *GSTT1* null genotype was observed in both presence and absence of cigarette smoke exposure.

Table 32. Multivariate logistic regression model for the combined risk of low birthweight and preterm delivery (<2500 g and <37th week gestation) conferred by maternal/newborn *GSTT1* and maternal smoking in the 3rd trimester

Smoking during 3rd trimester	*GSTT1* genotype		N	Proportion of <2500g and <37th GW		OR (95% CI)	P-value
	Maternal	Newborn		Observed	Expected		
No	Present	Present	453	0.0353	0.0308	1.0	
		Null	89	0.0449	0.0269	1.28 (0.42-3.94)	0.66
	Null	Present	106	0.0189	0.0550	0.53 (0.11-2.32)	0.40
		Null	63	0.1111	0.1080	3.41 (1.35-8.66)	0.010
Yes	Present	Present	148	0.0541	0.0678	1.56 (0.65-3.72)	0.316
		Null	27	0.00	0.0594		
	Null	Present	44	0.2045	0.1174	7.02 (2.89-17.04)	<0.001
		Null	24	0.2083	0.2176	7.19 (2.38-21.68)	<0.001

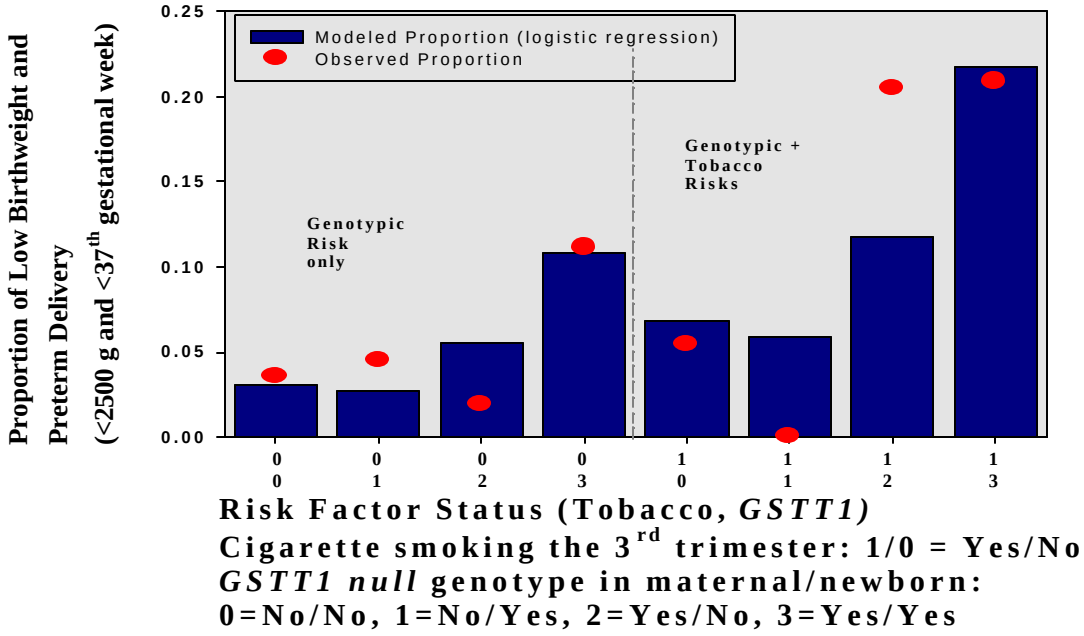

Figure 8. Combined association of maternal cigarette smoking during the 3rd trimester and combination of maternal/newborn *GSTT1* genotype with proportion of low birthweight and preterm delivery (<2500 g and <37th week gestation)

3.10.4 Multiple logistic regression analysis for SGA in term (≥ 37th week gestation) infants

For SGA in term infants, maternal/newborn *GSTT1* genotype alone did not confer a significant effect on the risk of fetal growth retardation by univariate logistic regression analysis (Table 33). However, a significant association of *GSTT1* genotype was observed in combination with maternal ethnicity or cigarette smoking in the 3rd trimesters. Among non-smokers, the presence of at least one risk factor: African American ethnicity or maternal or newborn *GSTT1* null

genotype, significantly increased the proportion of SGA compared with the non-exposed group (non-smoking Caucasians with *GSTT1* present genotype). Moreover, the presence of more than two risk factors dramatically increased the proportion of SGA. The highest proportion (0.294) was observed among African American smokers with maternal *GSTT1* null genotype (OR=9.89, p<0.001).

Table 33. Multivariate logistic regression model for the combined effects of maternal ethnicity, cigarette smoking during the 3rd trimester, and maternal or newborn *GSTT1* genotype, on SGA in term (≥ 37th week gestation) infants

Ethnicity	Smoking during 3rd trimester	*GSTT1* Genotype	N	Observed proportion of SGA	OR (95% CI)	P-value
		Maternal:				
Caucasian	No	Present	371	0.0404	1.0	
		Null	114	0.0351	0.863 (0.28-2.62)	0.80
African American	No	Present	187	0.1016	2.68 (1.33-5.41)	0.006
		Null	67	0.2388	7.45 (3.47-15.97)	<0.001
Caucasian	Yes	Present	149	0.1879	5.49 (2.84-10.63)	<0.001
		Null	47	0.1064	2.83 (0.98-8.17)	0.055
African American	Yes	Present	60	0.2167	6.56 (2.94-14-65)	<0.001
		Null	17	0.2941	9.89 (3.09-31.68)	<0.001
		Newborn:				
Caucasian	No	Present	392	0.0408	1.0	
		Null	92	0.0326	0.79 (0.23-2.78)	0.716
African American	No	Present	192	0.1354	3.68 (1.92-7.04)	<0.001
		Null	62	0.1452	3.99 (1.68-9.49)	0.002
Caucasian	Yes	Present	161	0.1677	4.74 (2.47-9.06)	<0.001
		Null	35	0.1714	4.86 (1.77-13.37)	0.002
African American	Yes	Present	56	0.2500	7.83 (3.57-17.17)	<0.001
		Null	21	0.1905	5.53 (1.67-18.33)	0.005

As shown Table 34 and Figure 9, the same sets of risk factors were observed in multivariate logistic regression analysis for SGA as were identified in the low birthweight infants (<2500g).

The combination of maternal/newborn *GSTT1* genotype modulated the proportion of growth-retarded infants in term births (≥37 week gestation).

Table 34. Multivariate logistic regression model for modulation of combined risk of SGA in term (≥37th week gestation) infants conferred by maternal smoking in the 3rd trimester and maternal/newborn *GSTT1* genotype

Ethnicity	Smoking during 3rd trimester	*GSTT1* genotype		N	Proportion of SGA		OR (95% CI)	P-value
		Maternal	Infant		Observed	Expected		
Caucasian	No	Present	Present	316	0.0411	0.0469	1.0	
			Null	54	0.0370	0.0421	0.896 (0.20-4.09)	0.888
		Null	Present	76	0.0395	0.0633	0.96 (0.27-3.45)	0.947
			Null	38	0.0263	0.0530	0.63 (0.08-4.95)	0.661
African American	No	Present	Present	150	0.1000	0.1128	2.59 (1.20-5.59)	0.015
			Null	37	0.1081	0.1020	2.83 (0.81-9.17)	0.084
		Null	Present	42	0.2619	0.1487	8.27 (3.42-20.02)	<0.001
			Null	25	0.2000	0.1264	5.83 (1.89-17.97)	0.002
Caucasian	Yes	Present	Present	129	0.1860	0.1366	5.32 (2.62-10.84)	<0.001
			Null	20	0.2000	0.1238	5.83 (1.71-19.90)	0.005
		Null	Present	32	0.0938	0.1785	2.41 (0.65-8.95)	0.189
			Null	15	0.1333	0.1525	3.59 (0.73-17.56)	0.115
African American	Yes	Present	Present	47	0.2340	0.2903	7.12 (2.97-17.07)	<0.001
			Null	13	0.1538	0.2676	4.23 (0.85-21.11)	0.078
		Null	Present	9	0.3333	0.3597	11.65 (2.62-51.86)	0.001
			Null	8	0.2500	0.3176	7.77 (1.43-42.27)	0.018

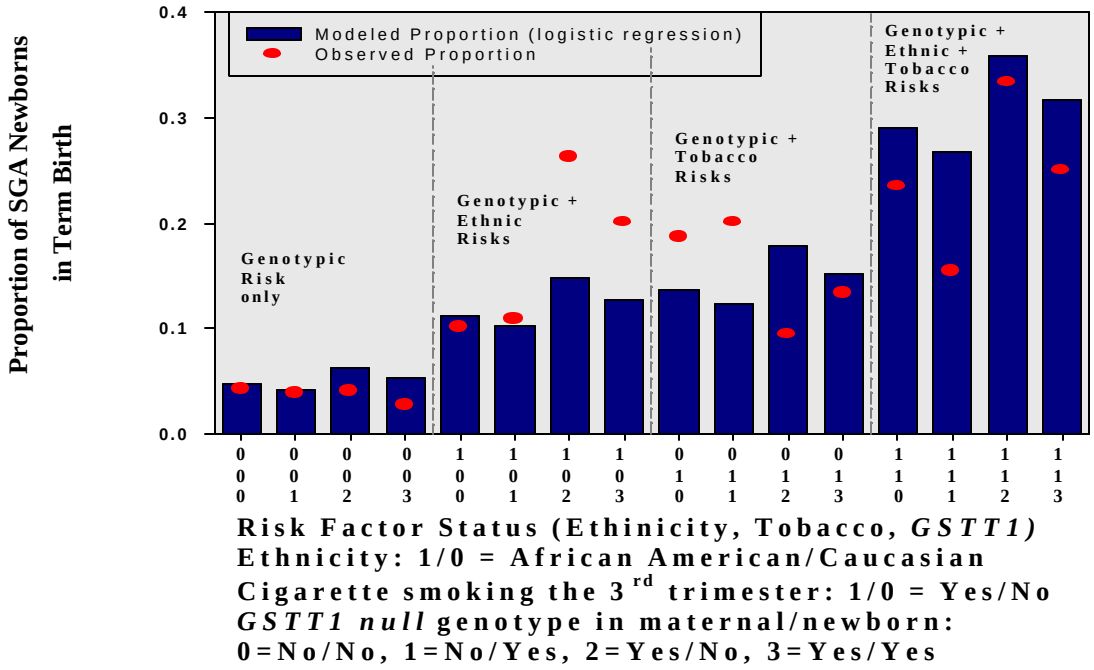

Figure 9. Combined association of maternal ethnicity, cigarette smoking during the 3rd trimester and combination of maternal/newborn *GSTT1* genotypes with proportion of SGA in term (>37th week gestation) infants

3.11 Univariate linear regression analyses

Univariate linear regression was performed to evaluate the associations of maternal demographic, lifestyle behavioral and maternal/newborn genetic factors with birthweight and also gestational week. The analyses were performed using of birthweight and gestational week as continuous variables. The predictor variables included: 1) maternal demographic factors such as age, family income, education level; 2) maternal lifestyle/exposure in early and late

pregnancies, active/passive cigarette smoke exposure, alcohol use, and the 3rd trimester risk factors; and 3) maternal/newborn genetic factors, *CYP1A1, CYP2E1, GSTM1, GSTT1* and *NAT2** genetic polymorphism frequencies data (Table 35).

The key results of this analysis are: African American infants were significantly smaller than Caucasian infants, with a mean reduction in birthweight of approximately 230g ($p<0.001$).

The greatest reduction in mean birthweight was observed among infants of teenage women. Early teenage women (≤15 years old) delivered infant weighing an average of 320 g less than infants of older women (21-30 years old) (referent group). Similarly, late teenage women (16-20 years old) delivered infants weighing 119.5 g less than infants average in the referent group ($p=0.002$). A significant positive correlation was observed between the mean birthweight (12.3 g/year old: SE, 2.6) and maternal age ($p<0.001$).

Compared to the AGA group, a reduction in mean birthweight was also found among women with lower family income (-203.3 g, -181.6 g, and –105.4 g) for <$10K, $10K-<$20K, and $20K-<$35K, respectively. Moreover, women with lower education also had significantly lower birthweight infants than women with higher education. The mean birthweight reduction observed among women with GED, high school graduate, and some college degree was –310.3 g, -231.2 g, and –217.4 g, respectively.

A reduction of the mean birthweight (-101.4 g, -63.8 g) was also observed if mothers were either actively or passively exposed to cigarette smoke during early pregnancy. A stronger

association and greater reduction of the mean birthweight (-228.6 g, -163.0 g) was observed if the mother actively smoked or was passively exposed to cigarette smoke during the third trimester. Moreover, a significant reduction of the mean gestational week (-0.24: SE, 0.1) was observed among infants of women who smoked during the 3rd trimester (p=0.05). In addition, a dose-response association was observed between the 3rd trimester exposure risk group and the reduction of mean birthweight (-45.4 g, -146.8 g) for the medium risk and high risk categories, respectively.

Lastly, a significant reduction of mean birthweight (-89.6 g: SE, 37) and mean gestational week (-0.25 week: SE, 0.1) was observed among the group with maternal *GSTT1* null genotype (p=0.018, p=0.049), respectively. Moreover, a significant reduction of the mean birthweight (-98.3 g: SE, 36) was found among the group with newborn *CYP1A1*2A,*3* genotype (p=0.007).

Table 35. Univariate linear regression summary for the effect of maternal/newborn demographic, behavior and genetic factors on birthweight and gestational week

Risk factor	N	Birthweight (g)		Gestation (week)	
		β (SE)	P-value	β (SE)	P-value
Maternal demographics					
Ethnicity					
African American	376	-230.0 (34.5)*	<0.001	-0.21 (0.1)	0.072
Caucasian	772	Referent		Referent	
Maternal age (years)					
≤15	11	-320.7 (168)	0.058	-0.16 (0.5)	0.776
16-20	318	-119.5 (38)*	0.002	-0.11 (0.1)	0.418
21-30	567	Referent		Referent	
≥31	252	67.9 (41)	0.106	-0.20 (0.1)	0.161
Continuous variables (13-45 years)	1148	12.3 (2.6)*	<0.001	-0.008 (0.001)	0.38
Family income					
<$10K	266	-203.3 (46)*	<0.001	0.15 (0.2)	0.333
$10K-<$20K	216	-181.6 (48)*	<0.001	-0.07 (0.2)	0.682
$20K-<$35K	157	-105.4 (54)	0.051	0.010 (0.2)	0.958
≥$35K	314	Referent		Referent	
Don't know	195	-226.2 (50)*	<0.001	-0.11 (0.2)	0.514
Maternal education					
< 12 grade	224	-310.3 (50)*	<0.001	-0.24 (0.2)	0.175
High school diploma	410	-231.2 (44)*	<0.001	-0.22 (0.2)	0.142
Some collage	269	-217.4 (48)*	<0.001	-0.13 (0.2)	0.439
BS or greater	245	Referent		Referent	
Cigarette exposure in early pregnancy					
Active					
No	616	Referent		Referent	
Yes	532	-101.4 (32)*	0.002	0.08 (0.1)	0.467
Never	605	Referent		Referent	
Less than a pack/day	261	-113.7 (41)*	0.006	0.076 (0.1)	0.585
Less than 2 pack/day	211	-86.3 (44)	0.052	0.128 (0.1)	0.392
More than 2 pack/day	71	-214.5 (69)*	0.002	-0.337 (0.2)	0.152
Continuous variable (0-50 cigarettes/day)	1148	-3.4 (2.2)	0.133	<0.001 (0.007)	0.997
Passive					
No	750	Referent		Referent	
Yes	398	-63.8 (34)	0.066	0.08 (0.1)	0.478
<3.5 h/week	750	Referent		Referent	
3.5-48 h/week	272	-51.1 (39)	0.196	0.06 (0.1)	0.631
>48 h/week	126	-91.1 (53)	0.091	0.123 (0.2)	0.497
Continuous variable (0-168 h/week)	1144	-1.18 (0.6)*	0.047	0.001	0.473

Table 35 cont.

Alcohol use					
No	169	Referent		Referent	
Yes	979	40.6 (46)	0.383	0.13 (0.2)	0.421
Continuous variable (0.41-1281 g)	1123	0.002 (0.1)	0.985	<0.001	0.090
Exposure during 3rd trimester					
Smoking					
No	798	Referent		Referent	
Yes	305	-228.6 (37)*	<0.001	-0.24 (0.1)*	0.05
Never	798	Referent		Referent	
Less than a pack/day	171	-236.1 (46)*	<0.001	-0.34 (0.2)	0.034
Less than 2 pack/day	87	-199.8 (62)*	0.001	0.29 (0.2)	0.177
More than 2 pack/day	47	-254.4 (82)*	0.002	-0.53 (0.3)	0.059
Continuous variable (0-40 cigarettes/day)	1103	-13.9 (3.2)*	<0.001	-0.012 (0.01)	0.25
Passive					
No	782	Referent		Referent	
Yes	319	-163.0 (36)*	<0.001	-0.05 (0.1)	0.57
<3.5 h/week	782	Referent		Referent	
3.5-48 h/week	231	-168.7 (41)*	<0.001	-0.02 (0.1)	0.903
>48 h/week	88	-148.1 (62)*	0.018	0.09 (0.2)	0.673
Continuous variable (0-168 h/week)	1101	-2.0 (0.7)*	0.005	0.001 (0.002)	0.83
Alcohol use					
No	1002	Referent		Referent	
Yes	99	-7.0 (59)	0.906	0.11 (0.12)	0.37
Continuous variable (0.08-640 g)	1096	-0.7 (0.9)	0.426	<0.001	0.91
Risk group					
Low risk	305	Referent		Referent	
Medium risk	721	-45.4 (38)	0.236	0.07 (0.1)	0.581
High risk	77	-146.8 (71)*	0.040	-0.02 (0.2)	0.918
Maternal genotype					
CYP1A1					
**1/*1*	767	Referent		Referent	
at least one copy of **2A* or **3*	380	-44.9 (35)	0.20	-0.1 (0.1)	0.4
CYP2E1					
**1/*1*	1067	Referent		Referent	
at least one copy of **5B*	81	-84.0 (64)	0.192	-0.28 (0.2)	0.19
GSTM1					
Present	614	Referent		Referent	
Null	534	63.1 (33)	0.056	0.25 (0.1)*	0.024
GSTT1					
Present	855	Referent		Referent	
Null	293	-89.6 (37)*	0.018	-0.25 (0.1)*	0.049
*NAT2**					
Fast	618	Referent		Referent	
Slow*	529	28.0 (33)	0.398	0.08 (0.1)	0.46

Table 35 cont.

Newborn genotype					
CYP1A1					
**1/*1*	811	Referent		Referent	
at least one copy of **2A* or **3*	335	-98.3 (36)*	0.007	-0.11 (0.1)	0.37
CYP2E1					
**1/*1*	1070	Referent		Referent	
at least one copy of **5B*	78	-43.9 (65)	0.503	-0.09 (0.2)	0.68
GSTM1					
Present	605	Referent		Referent	
Null	543	54.2 (33)	0.101	0.18 (0.1)	0.11
GSTT1					
Present	894	Referent		Referent	
Null	253	-65.0 (39)	0.102	-0.17 (0.1)	0.208
*NAT2**					
Fast	598	60.2 (33)*	0.07	0.15 (0.1)	0.17
Slow	548	Referent		Referent	

* Indicates statistically significant difference at p= 0.05

3.12 Multivariate linear regression analysis

Multivariate linear regression analysis was performed to further evaluate the role of maternal/newborn phase I/II metabolic enzyme genetic polymorphisms on adverse reproductive outcomes such as reduction of birthweight and shortened gestational week. The significant predictor variables that were found in the univariate linear regression analyses were tested in multivariate linear regression models. If these predictor variables did not reached significance, they were then removed from the model.

3.12.1 Multivariate linear regression for birthweight

As shown in Table 36, the combination of ethnicity, maternal smoking during the 3rd trimester and maternal/newborn *GSTT1* null genotype were significantly associated with both birthweight reduction and shortened gestational weeks. Much greater birthweight reductions were observed among the groups with *GSTT1* null in maternal and newborns genotypes compared with the group with *GSTT1* present. The greatest reduction in birthweight and gestational week were found among African American smokers with *GSTT1* null genotype with (-570g: SE, 117, $p<0.001$) and (-1.10 week: SE, 0.4, $p=0.007$), respectively.

Table 36. Multivariate linear regression summary for the effects of ethnicity, maternal smoking in the 3rd trimester and maternal or newborn *GSTT1* genotype, on birthweight

Ethnicity	Smoking during 3rd trimester	*GSTT1* genotype	N	β (SE) of birthweight (g)	P-value	β (SE) of gestation (week)	P-value
		Maternal					
Caucasian	No	Present	398	Referent	0.654	Referent	
		Null	123	24.8 (55)	<0.001	-0.07 (0.2)	0.713
African American	No	Present	200	-235.3 (46)	<0.001	-0.15 (0.2)	0.362
		Null	77	-372.9 (66)	<0.001	-0.37 (0.2)	0.108
Caucasian	Yes	Present	159	-200.3 (50)	<0.001	0.05 (0.2)	0.774
		Null	58	-472.2 (75)	<0.001	-0.63 (0.3)	0.016
African American	Yes	Present	66	-389.9 (71)	<0.001	-0.46 (0.2)	0.066
		Null	22	-570.0 (117)	<0.001	-1.10 (0.4)	0.007
		Newborn					
Caucasian	No	Present	418	Referent		Referent	
		Null	102	-18.8 (59)	0.753	-0.008 (0.2)	0.967
African American	No	Present	204	-254.5 (46)	<0.001	-0.11 (0.2)	0.486
		Null	73	-360.1 (68)	<0.001	-0.43 (0.2)	0.070
Caucasian	Yes	Present	178	-273.9 (48)	<0.001	-0.13 (0.2)	0.432
		Null	39	-317.3 (90)	<0.001	-0.05 (0.3)	0.879
African American	Yes	Present	63	-450.3 (72)	<0.001	-0.46 (0.3)	0.070
		Null	25	-426.7 (111)	<0.001	-1.0 (0.4)	0.012

Table 37 presents the combined association of ethnicity, maternal smoking during the 3rd trimester and maternal/newborn *GSTT1* genotype with infant birthweight and gestational week reduction. A significant reduction of birthweight and gestational week were observed among the groups which carried the *GSTT1* null genotype in mother, newborn or both compared with the group with both mother and newborn carried the *GSTT1* positive genotype. The greatest reduction in infant birthweight was observed among the group where both mother and newborn were *GSTT1* null genotype in the presence or absence of maternal smoking in both African Americans and Caucasians. A similar phenomenon was found in infant gestational age where

maternal/infant pairs of combined *GSTT1* null genotype were at the highest risk for gestational week reduction. The greatest reduction of gestational age (-1.51: SE, 0.5) was observed among the highest risk group that both mother and newborn carried *GSTT1* null genotype and the mothers were African Americans who smoked during the last trimester (p=0.008).

Table 37. Multivariate linear regression summary for combined risk of reduction of infant birthweight and gestational week conferred by maternal smoking in the 3rd trimester and maternal/newborn *GSTT1* genotypes

Ethnicity	Smoking during 3rd trimester	*GSTT1* genotype		N	Birthweight (g)		Gestation (week)	
		Mother	Bany		β (SE)	P-value	β (SE)	P-value
Caucasian	No	Present	Present	337	Referent		Referent	
			Null	60	-39.8 (75)	0.597	0.04 (0.2)	0.867
		Null	Present	81	19.5 (66)	0.769	-0.04 (0.2)	0.848
			Null	42	20.5 (87)	0.816	-0.10 (0.3)	0.733
African American	No	Present	Present	161	-238.4 (51)	<0.001	-0.25 (0.2)	0.163
			Null	39	-248.1 (90)	0.006	0.30 (0.3)	0.337
		Null	Present	43	-296.8 (87)	0.001	0.36 (0.3)	0.230
			Null	34	-480.4 (96)	<0.001	-1.3 (0.3)	<0.001
Caucasian	Yes	Present	Present	138	-218.3 (54)	<0.001	0.02 (0.2)	0.932
			Null	21	-119.9 (120)	0.321	0.33 (0.4)	0.438
		Null	Present	40	-449.1 (89)	<0.001	-0.68 (0.3)	0.029
			Null	18	-539.5 (130)	<0.001	-0.50 (0.4)	0.266
African American	Yes	Present	Present	52	-399.7 (80)	<0.001	-0.42 (0.2)	0.129
			Null	14	-376.6 (146)	0.010	-0.56 (0.5)	0.273
		Null	Present	11	-667.9 (164)	<0.001	-0.69 (0.5)	0.225
			Null	11	-481.9 (164)	0.003	-1.51 (0.5)	0.008

3.12.2 Multivariate linear regression analysis for the 3rd trimester exposure risk group

Multivariate linear regression analyses were performed to determine the effect of the 3rd trimester exposure risk group factors along with other maternal demographic, lifestyle and maternal/newborn genetic factors on the infant birthweight reductions. The risk factors, which

were identified by their association with birthweight in univariate linear regression analyses, were tested in a multivariate linear regression model. If the association did not reach a statistically significant level, the factor was removed from the model. The combined associations of ethnicity, the 3rd trimester risk group and maternal *GSTT1* genotype on infant birthweight were examined. Since maternal education level was significantly associated with the birthweight in the multivariate linear regression model in the presence of the 3rd trimester risk group, it was adjusted in the analyses.

As shown in Table 38, the greatest infant birthweight reduction was observed among *GSTT1* null genotype in the higher level of the risk group. The greatest birthweight reduction was found among the group of African Americans, high-risk group and maternal *GSTT1* null genotype (-649.3 g: SE, 388). However, it did not reach statistical significance (α=0.05)(p=0.095) although there are very few subjects in this combined subgroup.

Table 38. Multivariate linear regression summary for the effect of ethnicity, the 3rd trimester risk group, and maternal *GSTT1* genotype on infant birthweight

Ethnicity	The 3rd trimester risk group	*GSTT1* genotype	N	β (SE) of birthweight (g)	P-value
		Maternal			
Caucasian	Low risk	Present	119	Referent	
		Null	50	-2.7 (91)	0.976
African American	Low risk	Present	103	-260.6 (73)	<0.001
		Null	33	-344.2 (107)	0.001
Caucasian	Medium risk	Present	395	-97.5 (56)	0.088
		Null	108	-146.2 (72)	0.044
African American	Medium risk	Present	154	-279.0 (66)	<0.001
		Null	64	-445.3 (84)	<0.001
Caucasian	High risk	Present	43	-99.5 (96)	0.305
		Null	23	-507.6 (124)	<0.001
African American	High risk	Present	9	-415.5 (188)	0.028
		Null	2	-649.3 (388)	0.095

Table 39 summarizes the combined effects of ethnicity, the 3rd trimester risk group and maternal and newborn *GSTT1* genotypes on infant birthweight reduction. Greater birthweight reductions were observed among the group in which both mother and newborn were of *GSTT1* null genotype compared with either one being *GSTT1* null genotype. The greatest reduction of infant birthweight was found among African Americans whom both maternal and infant of *GSTT1* null genotypes (the high risk group) compared with Caucasians with both maternal and infant carried *GSTT1* present genotypes (the low risk group) (-846.7g: SE, 548). However, this association was not statistically significant (p=0.123).

Table 39. Multivariate linear regression summary for combined association of ethnicity, the 3rd trimester risk group and maternal/newborn *GSTT1* genotypes on infant birthweight

Ethnicity	The 3rd trimester risk group	*GSTT1* genotype		N	Birthweight (g)	
		Maternal	Newborn		β (SE)	P-value
Caucasian	Low risk	Present	Present	99	Referent	
			Null	20	-189.3 (133)	0.157
		Null	Present	36	-75.4 (106)	0.478
			Null	14	70.0 (155)	0.654
African American	Low risk	Present	Present	88	-317.1 (80)	<0.001
			Null	15	-146.0 (151)	0.335
		Null	Present	18	-281.8 (139)	0.044
			Null	15	-488.5 (151)	0.001
Caucasian	Medium risk	Present	Present	338	-137.5 (62)	0.028
			Null	56	-90.3 (91)	0.323
		Null	Present	68	-161.0 (85)	0.061
			Null	40	-206.9 (102)	0.043
African American	Medium risk	Present	Present	117	-291.6 (74)	<0.001
			Null	37	-370.6 (105)	<0.001
		Null	Present	35	-456.6 (107)	<0.001
			Null	29	-501.2 (115)	<0.001
Caucasian	High risk	Present	Present	38	-153.2 (104)	.0.141
			Null	5	36.1 (250)	0.885
		Null	Present	17	-474.4 (143)	0.001
			Null	6	-723.5 (229)	0.002.
African American	High risk	Present	Present	8	-405.5 (200)	0.044
			Null	1	-780.7 (548)	0.155
		Null	Present	1	-515.5 (548)	0.348
			Null	1	-846.7 (548)	0.123

3.13 Summary

These analyses clearly demonstrate that maternal/newborn phase I/II metabolic enzyme polymorphism can interact with other demographic and lifestyle risk factors such as ethnicity and cigarette smoke exposure to modulate individual risk of adverse reproductive outcomes (low birthweight, preterm delivery and growth retardation). As we anticipated from the results of other reproductive epidemiology, adverse reproductive outcomes in this study have strongly

associated with ethnicity and maternal smoking, especially during the last trimester. Interestingly, maternal and also newborn *GSTT1* genotypes were significantly associated most of those adverse reproductive outcomes**.** Moreover, the clear association of additive risk of maternal smoking during the last trimester and maternal/newborn *GSTT1* null genotype was observed for all adverse reproductive outcomes evaluated in this study.

4 DISCUSSION

The objective of this study was to determine if maternal/newborn phase I/II metabolic enzyme genetic polymorphisms contribute to individual risk of low birthweight, fetal growth retardation and/or preterm delivery. This study had several unique features. It is one of only a few published studies to investigate the association of phase I/II metabolic enzyme genetic polymorphisms on low birthweight, preterm delivery and SGA in term. It is the first study to evaluate the impact of not only maternal genotypes but also newborn genotypes as a maternal/newborn combined unit at risk of these adverse reproductive outcomes. It is based on a large and sociodemographically diverse population of pregnant women in Pittsburgh for whom epidemiological and clinical data were collected with a validated questionnaire using consistent methods by trained staff. This PEPP study was designed to evaluate the effects of maternal lifestyle variables and exposure on newborns at the molecular level using multiple biomarkers. This population of healthy pregnant women provided an opportunity to evaluate gene-environment interactions without substantial other medical confounders.

4.1 Low birthweight analyses

This study produced several important findings. In univariate analyses, maternal demographic factors including ethnicity, age, family income and education level were significantly associated with low birthweight (<2500 g). Women who were African American in their early teens, from low family income homes, and less educated were at increased risk for having a low birthweight infant. Similar results have been reported in recent studies[8, 40, 199,

200]. Several studies have suggested that these demographic factors are associated with maternal populations with health related disadvantages such as lack of prenatal care, poor nutrition, substance abuse including cigarette smoking and alcohol use during pregnancy, and other exposures [36, 39]. In multivariate regression analyses, only maternal ethnicity had a significant association with low birthweight. It is likely that the other demographic factors identified in the univariate analyses were highly correlated with ethnicity or maternal cigarette smoking history. For instance, African American women were more likely to be from lower family income groups or the mothers who continued to smoke were more likely to be less educated. The significant difference in birthweight between African Americans and Caucasians remained after consideration of all of the other risk factors evaluated in the study. African American infants were, on average, 230 g lighter at birth compared with the Caucasian infants, a value similar to that previously reported [8]. The OR of low birthweight for African American infants was 1.74. This ethnic might be differenced associate with socioeconomic status. A greater number of African American women in the study were of lower socioeconomic status by both family income and maternal education level measures, compared with Caucasians. There may also be some specific cultural differences, lifestyles and environmental exposures among the African American subjects that result in this reduction of infant birthweight [45, 49].

Both active and passive cigarette smoke exposure during the 3rd trimester were significantly associated with low birthweight in the univariate analyses. The mean birthweight reduction was approximately 228.6 g for active maternal smoking and 163.0 g for passive exposure. For every cigarettes smoked per day during the 3rd trimester, birthweight decreased approximately 13.9 g. Similarly, each increase by one hour of passive exposure per week during

this period reduced birthweight by approximately 2 g (Table 35). These results confirm similar observations in previously published studies in a range of 24-189 g birthweight reduction [30, 83, 201-203].

Maternal *CYP1A1* and *GSTT1* genetic polymorphisms alone were significantly associated with low birthweight in the presence of cigarette smoke exposure. Moreover, a statistically significant association of newborn *CYP1A1* genotype alone with low birthweight was found. These results agreed with a recent previous study by Wang et al. [203]. They reported a significant association of maternal *CYP1A1*2A* and *GSTT1* null genotypes with birthweight reduction. The mothers who continued smoking during pregnancy with at least one *CYP1A1*2A* allele increased the risk of having a low birthweight infant (OR=3.2) compared with the non-smoking mother with homozygous *CYP1A1*1* genotype. Similarly, the mothers who continued to smoke during pregnancy and were of *GSTT1* null genotype, had an increased risk of a low birthweight infant (OR=3.5) compared with non-smoking mothers with the *GSTT1* present genotype [203]. However, in the PEPP study, nether maternal nor newborn *CYP1A1* genotypes did not reach statistically significance for low birthweight in multiple regression analyses.

In our final regression model, maternal ethnicity, cigarette smoking during the 3rd trimester and maternal *GSTT1* genotype were all significantly associated with low birthweight. This is partially consistent with the findings of Wang et al. [203], who found that maternal *GSTT1* null genotype significantly modified the association between maternal cigarette smoking and infant birthweight in a Boston, MA population. We failed to observe the association of *CYP1A1*2A* and low birthweight in the multiple regression analyses. This might result from the

different ethnic distributions of the study population and the resulting differing *CYP1A1*2A* allele in this frequencies. In the Boston study, approximately 49.1% of the population was African American, only 16.5% of Caucasians, over 22.8% of Hispanic and more than 11.6% of other ethnical groups, while in the PEPP study, roughly 1/3 of the population was African Americans, 2/3 was Caucasians. A higher frequency *of CYP1A1*2A* has been observed in Asian populations and a lower frequency in African American populations [155, 156]. In addition, we screened the *CYP1A1*2A* and the *CYP1A1*3* polymorphism in the PEPP study population. *CYP1A1*3* is an African American specific polymorphism and approximately 30% of our study population was African American. Therefore, this might lead to conflicting results. The *CYP1A1*2A* polymorphism has been extensively studied for an association of the potential for activation of pro-carcinogenic compounds forming adducts and promotion of cancer development [134, 204-206]. It is also known that *CYP1A1*2A* is linked with the *CYP1A1*2C* polymorphism that affects CYP1A1 enzymatic activity [154]. However, the linkage between *CYP1A1*2A* and *CYP1A1*2C* depends on ethnicity. High linkage between those two alleles was observed among Asian populations but not in African American or African populations [129, 155]. Thus, if the population has a high percentage of African Americans, screening only for the *CYP1A1*2A* allele might not be reliable and could explain the non-concordant results of the two studies.

Figure 3 illustrates a bevy finding of this study that there is increase in the proportion of expected low birthweight infants for associated with the additive of maternal and newborn risk factors (ethnicity, cigarette smoking and maternal or newborn *GSTT1* genotypes) to the model. Non-smoking Caucasian mothers with *GSTT1* present genotype were classified as the lowest risk

referent group. There was a slight increase in the proportion of low birthweight births infants among the maternal *GSTT1* null genotype group alone and further increased among African Americans. In the presence of maternal smoking, the expected proportion is dramatically elevated and a further increased risk was observed among the maternal *GSTT1* null genotype group. Approximately a 6-fold increase in the proportion of low birthweight was observed among the group with all three risk factors (African American ethnicity, maternal smoking during the 3rd trimester and maternal *GSTT1* null genotype) compared with infants of the referent group. A similar association was observed with the newborn *GSTT1* genotype analyses. These data clearly indicate a dose-response risk relationship between maternal and newborn *GSTT1* positive/null genotypes with the addition of each risk factor.

Importantly, this is the first study to evaluate not only maternal polymorphisms in phase I/II metabolic genes but also the contribution of independent of combined newborn polymorphisms on low birthweight as well. The greatest birthweight reduction was observed in both maternal and newborn with the *GSTT1* null genotype compared with other genotype combination pairs. The lowest risk group was defined as both maternal and newborn carrying the *GSTT1* positive genotype. The risk of low birthweight increased incrementally by adding newborn *GSTT1* null genotype alone, maternal *GSTT1* null genotype alone and both *GSTT1* null genotypes (Figure 6). In addition, a dose-response relationship of *GSTT1* genotype was observed with each addition of ethnicity and maternal smoking factors. Since there was no observations of low birthweight infants in two groups (Caucasian smokers and newborn *GSTT1* null genotype, and African American smokers and newborn genotype of *GSTT1* null). Overall,

the expected and observed proportions of low birthweight infants agreed well particularly in the groups with an absence of risk factors because of larger numbers of subjects in these groups.

4.2 Preterm delivery (<37th week of gestation) analyses

Unlike multiple risk factors for low birthweight, only one maternal demographic factor, maternal education level, was significantly associated with preterm delivery in the univariate analyses. Preterm delivery has been associated with maternal smoking during the 3rd trimester in previous studies [203, 207, 208]. Our analyses also demonstrated both maternal and newborn *GSTT1* null genotypes were significantly associated with preterm delivery. Our finding confirms the observation made in a previous study that maternal *GSTT1* null genotype frequency was significantly higher among the mothers with preterm delivery [203]. Wang et al. reported approximately a 2.1 week (SE=0.8) gestational age reduction if the mothers continued to smoke during pregnancy and carried the *GSTT1* null genotype (OR=2.8, 95% CI 1.1-7.8) compared with the non-smoking mothers with *GSTT1* present genotype. Our results showed a more moderate reduction in gestational age (0.25 weeks, SE=0.1, p=0.049) if the mother carried the *GSTT1* null genotype. The greatest reduction of gestational age was observed among African American smokers with *GSTT1* null genotype in both mothers and newborns (1.51 weeks, SE=0.5, p=0.008) (Table 37). In the absence of maternal smoking, the observed and expected proportion of preterm deliveries was similar among the different combinations of maternal/newborn *GSTT1* genotypes in the multivariable logistic regression model. However, the proportion was dramatically increased in a dose responsive manner among smokers. Thus, it is biologically plausible that without maternal tobacco smoke exposure, the *GSTT1* null genotype was not a risk factor. Furthermore, we observed that if both maternal and newborn pairs carried

the *GSTT1* null genotypes, there was a corresponding increase in susceptibility for preterm delivery. One previous study reported that *GSTT1* null was a high risk genotype for shortened gestation in the presence of low levels of maternal exposure to benzene [209]. In animal studies, benzene and other organic solvents result in delayed fetal development and reduced birthweight [210]. In human epidemiological studies, organic solvent exposures have a weak association with increasing risks of birth defects and spontaneous abortion [211]. The etiologies of preterm delivery are still unclear. It is clear that GSTT1 is responsible for detoxification of these toxins and we and other investigators have observed that the absence of GSTT1 significantly increases the risk of preterm delivery. This might be associated with the disturbance of blood circulation in the placenta. Several studies have reported that maternal cigarette smoke exposure dramatically affects placental vasculature. The observed reduced dimensions of fetal capillaries in villi may affect placental blood flow and the diminished area for exchange of gases and nutrients between the mother and the fetus will increase the risk of fetal undernourishment [212]. Our study suggested that not only the maternal detoxification process is important but also that differences in genetically determined fetal metabolism also contribute to the risk of preterm delivery.

Wang et al. [203] also reported a significant association of maternal *CYP1A1*2A* genotype with preterm delivery. A diverse of approximately 1.5 weeks of gestational age occurred if the mother continued to smoke during pregnancy and carried at least one *CYP1A1*2A* allele (OR=2.2, 95% CI 1.1-4.4) compared with non-smoking mothers homozygous for *CYP1A1*1*. However, our results did not confirm their finding. It might be because of the same factors previously mentioned in the low birthweight analyses. In addition, maternal *NAT2**

slow acetylator genotype was a protective factor for preterm delivery in our analyses. Since no clear mechanism for causing preterm delivery is known, the maternal *NAT2** genetic polymorphism may also represent a critical detoxification pathway. Wang et al. [203] did not report screening for *NAT2** polymorphisms.

4.3 Low birthweight and preterm delivery (<2500 g and <37th weeks of gestation) analyses

The risk factors associated with low birthweight and preterm delivery (premature birth) were somewhat similar to those observed in the preterm delivery and low birthweight infant groups alone. Maternal ethnicity, age and education level were significantly associated with both low birthweight and premature birth in univariate analyses. Early teenage (≤15 years old) African American mothers with lower educational level were more likely to have a premature infant. Our results are similar to those of previously published studies in that maternal age less than 15 years is associated with increased risk of adverse reproductive outcomes [40]. In addition maternal smoking during the third trimester was strongly associated with preterm delivery and low birthweight [30, 213]. Moreover, in out study, we also observed that maternal *GSTT1* null genotype was significantly associated with premature birth. Newborn *GSTT1* null genotype showed a trend towards increased risk but did not reach statistical significance (p=0.65). A similar pattern was observed for the maternal/newborn combined *GSTT1* null genotype and low birthweight/premature birth. Both maternal and newborn *GSTT1* genotype modulate the proportion of combined low birthweight and premature birth in the presence and absence of maternal smoking. The expected proportion of this adverse outcome among the non-smoking groups showed a slight increase in risk for each combination of maternal/newborn

GSTT1 null genotype. However, if the mother smoked during the last trimester, the expected proportion of low birthweight/premature birth dramatically increased among the groups where at least one of the pair carried the *GSTT1* null genotype. The largest proportion of low birthweight/premature birth infants was observed among the group with both mother and newborn of *GSTT1* null genotype in the presence of maternal smoking. These findings are consistent with a previous study [203] for a smaller population. In the previous study, Wang et al. [203] reported maternal *GSTT1* null genotype and the *CYP1A1*2A* allele were the high risk genotype for both low birthweight (<2500 g) and preterm birth (<37th weeks of gestation) infants. However, they did not analyze specifically for the combined group with less than 2500 g birthweight and less than 37th weeks of gestation.

4.4 SGA at term delivery (≥ 37th weeks of gestation) analyses

Small for gestational age (SGA) was defined as less than the 10th percentile of birthweight among term infants (≥37th weeks of gestation). Several maternal demographic factors were identified as significantly associated with SGA including maternal ethnicity, age, family income and education level in univariate analyses. Interestingly, African American mothers who were older than 31 years with low family income and lower educational level were more likely to have an infant with SGA. As discussed, the association of ethnicity, family income and educational level with other adverse reproductive outcomes might be related to medical care access disadvantages and other ethnic specific risk factors.

Overall, our analyses, older women (≥31 years old) have the highest risk of SGA. This might be the result of age-related increases in fetal chromosome aberrations and the presence of complicating maternal medical factors such as hypertension [32, 33].

Maternal smoking during the 3rd trimester but also in early pregnancy was strongly associated with SGA. Moreover, risk of SGA with passive cigarette smoke exposure during the third trimester was also statistically significant. Our findings are consistent with previous studies [29, 119]. Most growth retardation occurs after the 28th week of gestation, but it may occur earlier [20]. Maternal smoking in early pregnancy and even before the conception are known to impact adverse reproductive outcomes including SGA [214]. Cigarette smoke exposure in early pregnancy may result in DNA and chromosomal damage that may affect fetal structural development [20]. Fetal cell size dramatically increases after 32 weeks of gestation. Cigarette smoke exposure around this period inhibits depositions of fat and glycogen and cell growth [20, 30, 85].

Maternal and newborn *GSTT1* null genotype combinations significantly associated with SGA and other risk factors specifically maternal ethnicity and smoking during the 3rd trimester. A graduated increase in the expected proportion of SGA was observed by the incremental addition of these risk factors. In the absence of maternal smoking among Caucasians (low risk group), the expected SGA proportion was slightly increased if either mother/newborn or the pair together carried the *GSTT1* null genotype. The presence of at least two additional risk factors dramatically elevated the proportion of SGA. A dose-response relationship was observed among the combination mother/newborn pair *GSTT1* null genotype (Figure 8).

The combination of the third trimester exposure risk group and maternal/newborn *GSTT1* null genotype was significantly associated with SGA. Mothers in the high exposure group have a much higher risk of having a SGA infant. Among this same study population, Dr. Gordish reported that a significant elevation of maternal/newborn glycophorin A (*GPA*) locus somatic cell mutation frequency was observed among high exposure group compared with that in the low exposure group [197]. Maternal smoking was strongly associated with SGA but other maternal exposure factors such as consumption of alcohol, caffeine, and charbroiled meat, also affect DNA damage and other toxicity endpoints. The affect of these individual exposures alone in SGA risk do not reach statistical significance, but the sum of these exposures (represented by the third trimester exposure group) increases the risk of SGA (OR=2.22, p=0.045) (Table 26).

4.5 Risk factors associated with adverse reproductive outcomes

Maternal ethnicity, cigarette smoking during the 3rd trimester and maternal/newborn *GSTT1* genotype commonly appeared as risk factors for all of the adverse reproductive outcomes evaluated in this study. Maternal ethnicity appeared only if adverse reproductive outcomes were related to reduction of birthweight but not for shorted the gestational age.

4.6 Maternal smoking during the third trimester

Maternal cigarette smoking during the 3rd trimester was strongly associated with reduction of birthweight to cause low birthweight, shortened gestational age and infant growth retardation. This finding is consistent with the existing literature [215].

Maternal passive smoke exposure was not as strongly associated with these adverse reproductive outcomes as active smoking during this critical period. According to measured blood cortinine levels, non-smokers passively exposed to cigarette smoke generally have a 10-100 fold lower level than active smokers. This suggests that the biological effects of passive exposure should be correspondingly lower than those of active smoking. Environmental tobacco smoke is composed of a complex mixture of carcinogenic/mutagenic and toxic compounds contained in exhaled mainstream and sidestream smoke. Passive smoke exposure contains the same or much higher levels of these compounds. Thus, passive exposure may cause adverse effects as strongly as active smoking. It is difficult to separate women exposed only passively to cigarette smoke from active smokers. The women in our study population might have reported a smaller amount of cigarettes that they smoked during the 3rd trimester especially if those adverse reproductive outcomes occurred. Several existing studies evaluated accuracy of self-reported maternal cigarette smoke status during pregnancy. There are ethnic differences in how accurately they report; self-reported smoking status among minority groups were more likely to be misclassified.

On the other hand, physical measurements of cigarette smoke exposure, most commonly by cotinine measurement, may be modulated by *CYP2A6* polymorphisms. Nicotine is primly metabolized by *CYP2A6* in humans. The *CYP2A6 gene* is highly polymorphic with many effects on enzyme activity. Further disussion about the possible importance of *CYP2A6* is found in section 4.11.2.

4.7 Modulation by *GSTT1* genotype and susceptibility of adverse events

The biological mechanisms by which a genetic polymorphism modifies the toxic effects of cigarette smoke on adverse reproductive outcomes is not well understood. Reduction of birthweight caused by fetal growth retardation and shortening of gestational age may be due to disturbance of cell regulation caused by DNA adducts and damage. A number of epidemiological studies have evaluated the role of *GSTT1* metabolism, and the *GSTT1* genotypes, in a number of different diseases including cancer.

The most well documented examples of an association between chemical agents lack of GSTT1 and adverse events are a-vinyloxirane (aka 3,4-epoxybutene, MEB) and [2,2']bioxiranyl (aka 1,2:3,4-diepoxybutane, DEB) which induce DNA damage, resulting in chromosomal aberrations, micronuclei, and sister chromatid exchanges in a variety of systems [216-221]. MEB and DEB are reactive metabolites produced via oxidation of the 1,3-butadiene (BD) by CYPs. Epidemiological studies have evaluated the induction of leukemia caused by BD exposure. Resent studies had reported that MEB and DEB are substrates for GSTT1-1. Thus, mothers with *GSTT1* null genotype are more susceptible to BD induced DNA damage. It had been confirmed in in vitro studies that *GSTT1* null genotype was less protective against DEB genotoxic activities [191, 220, 222-226]. In addition, GSTT1-1 null subjects were more sensitive than GSTT1-1 positive subjects to sister chromatid exchanges caused by MEB [227]. Human lymphocytes which are treated with DEB have a higher mean frequency of genotoxic events such as micronuclei and chromosomal aberrations in *GSTT1* null subjects compared with *GSTT1* positives [218, 219, 221, 223].

Although the studies conducted *in vitro* suggest an important role of GSTT1-1 against the metabolites of BD, the processes occurring in humans exposed in vivo to BD are largely unknown. One study reported that the mean frequency of chromosomal aberrations was higher in peripheral blood lymphocytes of *GSTT1* null genotype subjects compared with that of *GSTT1* positives by BD exposure [228]. This result agreed with in vitro studies that *GSTT1* null individuals appear to be more susceptible to BD exposure. However, findings from several other studies were inconclusive. Other metabolic enzyme genotypes in addition to the *GSTT1* polymorphism may play an important role in further modulating the biological effects of BD exposure.

Similarly, human *GSTT1* is involved in the detoxification of Phenloxirane (aka styrene-7,8-oxide, SO), a metabolite of Phenylethene (aka styrene). In vitro studies with lymphocyte cultures with *GSTT1* null genotype had approximately 1.4-1.7 times greater frequency of sister chromatid exchanges per cell compared with *GSTT1* positive subjects [229]. It was concluded that although glutathione-*S*-transferase conjugation could be a minor metabolic pathway in vivo for SO or for BD, the high GSTT1-1 activity in erythrocytes may be important locally [162].

Several studies have evaluated the association of the *GSTT1* polymorphism and frequencies of in vivo genotoxic and cytotoxic events such as sister chromatid exchanges, chromosomal aberrations, and micronuclei in the absence of any specific source of exposure. Higher background frequencies of sister chromatid exchanges were observed among *GSTT1* null genotype compared with that among individuals with active *GSTT1* [191, 222, 230]. However this finding was not confirmed in other studies [223, 227]. In addition, the lowest mean

frequency of sister chromatid exchange was observed among non-smoking individuals with the *GSTT1* positive genotype as compared with the highest levels in *GSTT1* null smokers in a dose-response relationship [230]. *GSTT1* positive smokers and *GSTT1* null non-smokers had intermediate levels of sister chromatid exchange. Other studies suggested that *GSTT1* null genotype individuals are less susceptible to chromosome aberrations [229]. Several studies suggested that oxirane (aka ethylene oxide epoxide, EO), produced endogenously or inhaled with cigarette smoke, could be one of the genotoxic GSTT1-1 substrates responsible for the different spontaneous rate of sister chromatid exchanges and chromosome aberrations [230, 231].

PAHs are well-known carcinogens contained in cigarette smoke that are metabolized by GSTT1-1. However, it is not clear if the *GSTT1* polymorphism is associated with increased genotoxic or carcinogenic risk in smokers. Two studies reported that no differences were observed among *GSTT1* positive or null genotype individuals exposed to PAHs in levels of DNA single strand breaks, DNA protein cross links, DNA adducts, or sister chromatid exchanges [232, 233]. However, other studies reported that the GSTT1-1 positive genotype was associated with increased levels of excreted 1-hydroxypyrene glucuronide and the activity of GSTT1-1 in erythrocytes was inversely correlated with the DNA adducts in mononuclear leukocytes [232]. Thus, although several polycyclic aromatic hydrocarbons are metabolized by *GSTT1* this could represent only a minor metabolic pathway. Further investigation is necessary to better understand the role of *GSTT1* mediated metabolism in the elevation of adverse reproductive outcomes.

4.8 Elevation of DNA and protein adducts is positively correlated with DNA damage and other health effects including adverse reproductive outcomes

Previous studies have reported significantly higher levels of DNA and hemoglobin adducts among mothers who smoked compared with non-smoking mothers [132, 133, 189]. Moreover, cigarette smoke related specific amine adducts have been observed in smokers' placentas. Positive dose response relationships were observed between the number of cigarettes smoked and the level of smoking related adducts found in placenta and in cord blood.

Additional studies have demonstrated that DNA adduct levels in placenta and cord blood are negatively correlated with infant birthweight. In addition, these adducts were also found in fetal tissues [234]. The study demonstrated that maternal transfer of carcinogens present in cigarette smoke to fetal tissues (umbilical cord artery and vein) and showed that these tissues can metabolize the carcinogens to their DNA binding metabolites. Hatch et al. reported that significantly higher levels of PAH-derived adducts were found in 43% of placentas and spontaneously aborted fetal liver (27%) and lung (42%) [235]. Another study evaluated whether oxidative damage to DNA, as measured by 8-oxo-7,8 dihydro-2 deoxyguanosine, is increased with low birthweight and fetal growth retardation early in the third trimester [236]. Since there is a significantly higher incidence of hepatoblastoma in children with low birthweight, oxidative DNA damage (8-OHdG) was measured among those children with hepatoblastoma. However, no statistical association was observed between the level of oxidative DNA damage and incidence of hepatoblastoma and low birthweight [237]. An association between elevation of oxidative DNA damage and low birthweight and fetal growth retardation was inconclusive. In addition, Woods et al. reported increased spontaneous chromosome breakage was observed in blood and

fibroblasts of growth retarded infants [238]. An elevation in DNA and protein adducts in associated with DNA damage and risk of adverse reproductive outcomes.

4.9 Association of elevated DNA adducts in placenta and disruption of placental function

Extensive studies have been done to identify occupational and environmental exposures, including cigarette smoke, which increase DNA adduct levels in placenta. It has been hypothesized that DNA damage may influence blood circulation resulting in the inhibition of gas and nutrient supply to the fetus. This disfunctioning placenta might cause fetal growth retardation, low birthweight and preterm delivery. Since it is known that phase I/II metabolic enzyme are expressed in placenta [153, 250, 251], metabolic enzyme genetic polymorphisms in placenta may modulate the level of DNA adducts and level of damage to DNA and affect placental development and function.

4.10 Study limitations

4.10.1 Selection and size of the study population

In this study population, approximately 4.3% of singleton infants weighted less than 2500 g at the birth. The proportion of low birthweight infants in this study was less compared with that (6.0%) had reported in the National Vital Statistical Report for the year 2000. Women with high risks of adverse reproductive outcomes may not have agreed to participate to the study.

Moreover, these high potential risk mothers might not have qualified to participate in the PEPP study and were excluded at enrollment. In addition, these women may have been included in the study as a result of incomplete sample collection due to an unexpected delivery time or the development of complications of pregnancy. Finally, the PEPP follow up questionnaires data was not obtained for approximately 4% of the maternal subjects who either refused to participate or did not come to the clinic for delivery, resulting in missing data. This 4% of subjects may have included a disproportionate number of at risk women. Therefore, there may have been a subject selection bias, resulting in the failure to include certain risk factors for adverse reproductive outcomes.

4.10.2 Caffeine consumption

Quantification of caffeine intake during the last trimester was not reliable. In the follow-up questionnaire, consumption of soft drinks during the last trimester was not quantified according to the amount of caffeine each beverage contained. Similarly, the question for black tea consumption did not distinguish between caffeinated/decaffeinated tea. Thus it would be difficult to demonstrate a true association between caffeine consumed during pregnancy and low birthweight and preterm delivery in this study. In addition, only self-reported caffeine consumption was available in this study that might introduce misclassification of caffeine consumption.

4.11 Future directions

4.11.1 Collection of additional maternal demographic and clinical information

Other maternal demographic and lifestyle risk factors associated with low birthweight and preterm delivery have been identified but were not included in the analyses in this study such as parity, marital status, other substance abuse, nutritional status, and excessive physical activity. Some of this information was collected from individual women but were not available for to sufficient numbers of subject include in the analyses. Several categories of maternal clinical and pregnancy information is available to evaluate in future analyses. In addition, newborn length and head circumference data were also not available at the time of analyses but in the future, these could be abstracted from clinical records. These additional measures might be used to characterize growth retarded infants more accurately than using only birthweight.

4.11.2 *CYP2A6* polymorphism and nicotine metabolism

Approximately 80% of nicotine is metabolized to cotinine by CYP2A6. CYP2A6 appears to be the sole catalyst of nicotine catabolism at low physiologically relevant nicotine concentrations of 50 μM. At high nicotine concentrations (500 μM), other CYP isoforms, such as CYP2B6 also participate in nicotine oxidation [239]. In the general population, approximately less than 1% of individuals are classified as CYP2A6 poor metabolizers in Caucasians and up to 20% in Asians. Several genetic polymorphisms in this gene have been identified that diminish enzyme activity. Several pharmacogenetic studies reported that

individuals with the CYP2A6 gene deletion had only 15% of the levels of cotinine in their urine when compared with subjects having at least one active gene, after smoking an equal number of cigarettes [240]. Another study reported no detectable levels of cotinine in plasma was observed among individuals who were CYP2A6 poor metabolizers [241]. Thus, nicotine-cotinine metabolism is dependent on *CYP2A6* gene activity that is affected by genetic polymorphisms. It could cause misclassification of cigarette smoking status measured by cotinine level without considering the *CYP2A6* polymorphism. Since *CYP2A6* is the major enzyme responsible for nicotine metabolism, adverse reproductive outcomes caused by nicotine and/or its metabolites might be modulated by *CYP2A6* genetic polymorphisms. In addition, extensive studies have evaluated if *CYP2A6* poor metabolizers differ in their smoking habits from extensive metabolizers, and if this polymorphism is associated with increased risk of cigarette smoke associated tobacco related health effects such as preterm delivery, low birthweight and also lung cancer later in life. The CYP2A6 enzyme can activate several procarcinogens contained in cigarette smoke such as nitrosamines and aflatoxins [127]. One study reported that the allele frequency of the *CYP2A6* gene deletion among lung cancer patients was significantly lower than that among healthy controls in the Japanese population [242]. The CYP2A6 poor metabolizers are protected from the several procarcinogens in tobacco by diminished activation and less likely to be addicted to cigarette smoking.

4.11.3 *GSTP1* polymorphism-the most abundant enzyme observed in fetal liver

GSTP1 is known as the acidic form of the GST isoenzymes and is present only in the early fetal development similar to CYP3A7. The GSTP1 protein is detectable during pregnancy at the neonatal stage but it is not detectable in one month old and older infants' liver. In

addition, GSTP1 is the major GSTs in fetal liver (10-22 weeks of gestational age, 18.0-25.2 pmol/mg cytosol protein). GSTP1 expression decreases during the second and the third trimester to 5.0 pmol/mg cytosol protein [243]. In addition, Cossar et al. reported that GSTP1 represents the major GST isoenzyme in the developing lung [244].

GSTP1 is also known to be over expressed in pre-neoplastic and tumor tissues [245]. Extensive studies have been conducted to evaluate *GSTP1* as a marker for early detection of tumors including smoke-associated cancers. Moreover, GSTP1 plays a role in the detoxification pathway for PAHs such as benzo[*a*]pyrene, and chrysene.

Previous studies have demonstrated that the allelic variants of *GSTP1* gene affect the catalytic efficiency of the enzyme in the detoxification of carcinogenic diol epoxides of benzo[*a*]pyrene or structurally related PAHs [246]. In epidemiological studies, approximately 45% higher level of DNA adducts in breast tissues was observed among individuals with *GSTP1* risk genotype compared with individuals without the genotype [247]. One study reported a significant increase of single strand DNA breaks, chromosome aberrations and *HPRT* locus mutations in peripheral blood lymphocytes among individuals with the *GSTP1* risk genotype caused by styrene exposure [248]. Accordingly, newborn *GSTP1* genetic polymorphisms may modulate the susceptibility of adverse reproductive outcomes.

4.11.4 DNA repair enzyme polymorphisms and adverse reproductive outcome.

Among the several DNA repair pathways, nucleotide excision repair (NER) is the one of the most effective repair pathways in removing bulky DNA adducts caused by cigarette smoke

exposure. Epidemiological studies have evaluated associations of DNA repair capabilities and incidence of cancer and other health effects. One of the best and extreme examples for a direct relationship was observed among xeroderma pigmentosum patients. Xeroderma pigmentosum is known for the lack of NER and patients are unable to remove bulky adducts, resulting in a 1000-4000-fold increased risk of skin cancer at very young ages [249].

Similar to phase I/II metabolic enzymes, a number of genetic polymorphisms in NER genes and in genes involved in other repair pathways have been identified and evaluated for functional significance. Several epidemiological and also functional studies have been done to evaluate the modulation of cancer risk caused by DNA repair genetic polymorphisms [252, 253]. However, to date, these findings are inconclusive.

Adverse reproductive outcomes such as fetal growth retardation and premature delivery are strongly associated with maternal cigarette smoking that will increase the frequency of DNA damage including adducts formation. We found that risk of these adverse outcomes are modulated by maternal and also newborn metabolic enzyme detoxification activities. The events may also be modulated by newborn DNA repair capability. To date, currently no studies have been performed to evaluate the possible association between newborn DNA repair genetic polymorphisms and adverse reproductive outcomes.

4.12 Summary

Extensive epidemiological studies have been done to evaluate risk factors associated with adverse reproductive outcomes including low birthweight, preterm delivery and fetal growth

retardation that have higher risk of infant mortality and health problems in childhood and even early adulthood. Moreover several studies have been reported that women have higher probabilities of adverse reproductive outcomes and developing pregnancy complications if they were of low birthweight, or had growth retardation and premature birth themselves. Our results could be a useful strategy to decrease the incidence of these adverse reproductive outcomes by the identification of high risk pregnant and targeted interventions to reduce risk associated with modifiable lifestyle risk factors such as smoking during pregnancy. Identification of high risk mothers for adverse reproductive outcomes at early stages during the pregnancy is likely to improve infant survival and reduce health problems later in life.

5 CONCLUSIONS

The PEPP study was designed as a molecular epidemiological study to investigate the potential transplacental genotoxic impact of prenatal environmental exposures on levels of DNA damage observed in newborns by applying biomarkers of exposure, genetic susceptibility, and early biological effects. The goal of this dissertation research was to evaluate the impact of maternal and newborn phase I/II metabolic enzyme genetic polymorphisms on birthweight reduction caused by fetal growth retardation and premature birth as a result of shortened gestational age. These adverse reproductive outcomes have an increased infant mortality rate and are associated with a number of health problems at birth and even later in life. Extensive epidemiological studies have been conducted to identify maternal demographic, obstetric and lifestyle factors associated with these adverse events. However, little is known regarding genetic susceptibility to these outcomes.

Phase I/II metabolic enzymes play an important role in the activation and detoxification of xenobiotics we are exposed to daily. There are wide inter- and intra-individualities in xenobiotic and drug response and toxicity. This variability is in part attributable to genetic polymorphisms. Many molecular epidemiological studies have evaluated the association of metabolic enzyme genetic polymorphisms and susceptibility to disease, including cancer. We hypothesized that the amount of toxic substrates which reach the fetus may be modulated by maternal susceptibility factors (age, nutrition, parity, stress, disease state, phase I/II metabolic enzyme activities and other genetic factors), and involvement of placenta metabolism when the

mother was exposed to toxicants such as cigarette smoke. DNA damage and adverse reproductive outcomes observed in newborns may be further modulated by fetal susceptibility factors including phase I/II metabolic enzyme activities. Here, we examined the contribution of five metabolic enzyme genotypes in both maternal and newborn samples.

Several maternal demographic, lifestyle and maternal/newborn genetic factors were identified as significant risk factors for low birthweight, preterm delivery, premature birth and SGA using univariate analyses. The risk factors for each adverse reproductive outcome were similar but not completely identical. These results support the observation of Wilcox [5] that birthweight reduction and shortened gestational age are independent events.

Our results are consistent with previous findings that maternal cigarette smoking, especially during the last trimester, is strongly associated with the reduction of birthweight, shortened gestational age and fetal growth restriction. A dose response relationship was observed between the number of cigarettes smoked per day and the mean birthweight reduction. Passive cigarette smoke exposure during this period was also associated with those adverse reproductive outcomes.

Ethnicity was also identified as a significant risk factor for low birthweight newborns. African American women have a higher risk of having a low birthweight infant compared with Caucasians. However, low birthweight infants among African Americans were not associated with premature births. These women were more likely to be of lower socioeconomic status, which may lead to a higher risk of exposure to harmful environmental agents. Lower

socioeconomic status is often associated with poorer health care access, including a lack of prenatal care. Furthermore, African American specific genetic factors may predispose this ethnic group for adverse reproductive outcomes.

The association of maternal *GSTT1* genotype alone on SGA was not statistically significant but the likelihood of delivering an infant with SGA was significantly associated with the *GSTT1* null genotype in combination with other risk factors, i.e. maternal cigarette smoke exposure during the last trimester and ethnicity. Among low birthweight infants, the observed proportion of mothers with the *GSTT1* null genotype was significantly increased in the presence of exposure, in this case, maternal cigarette smoking during the last trimester. African American ethnicity and newborn *GSTT1* null genotype further increased the risk of low birthweight. A similar set of risk factors was observed in the analyses of preterm delivery and premature birth, however maternal ethnicity was no longer significant. Both maternal and newborn *GSTT1* null genotype was identified as the "high risk" genotype combination for birthweight reduction and shortened gestational age and the risk dramatically increased in the presence of maternal cigarette smoke exposure. These results were consistent with the recent findings of Wang et al. where maternal *GSTT1* genotype was significantly associated with low birthweight, preterm delivery and premature birth. Newborn genotype was not investigated by Wang et al. [188].

Therefore, both maternal genetic and exposure factors contribute significantly to adverse reproductive outcomes. These associations were significantly modified by newborn phase I/II metabolic enzyme genetic polymorphisms. A strong association was observed between maternal/newborn *GSTT1* genotype and low birthweight, preterm delivery and SGA in term

infants. The estimated association was significantly greater for *GSTT1* null genotype than for *GSTT1* positive in low birthweight, preterm delivery, <2500 g and <37th weeks of gestation, and SGA in term delivery infants by univariate logistic regression. Multivariate analyses showed that maternal *GSTT1* null genotype represents a greater risk factor for these adverse reproductive outcomes compared with newborn *GSTT1* null genotype alone. However, the combination of both maternal/newborn *GSTT1* null genotype is the greatest risk factor for all of the adverse effects analyzed in this study. The *GSTT1* null genotype alone was not significant risk factor for SGA. However, in combination with maternal ethnicity and presence of cigarette smoke exposure during the last trimester, maternal/newborn *GSTT1* null genotype significantly increased the risk of SGA.

Aside from observing that the frequency of the *NAT2** fast acetylator genotype was significantly higher among women with preterm delivery compared with women in AGA (p=0.032) in an univariate analysis, we failed to observe an association between the *CYP1A1, CYP2E1, GSTM1* and *NAT2** genotypes and risk of adverse birth outcomes. Insufficient power to detect gene-gene interactions is possible. It is also very likely that other genetic factors not yet identified, may be associated with adverse reproductive outcomes. For example, the role of fetal DNA repair capacity in modifying the adverse effect of maternal environmental exposures and maternal/newborn genetic interactions remain unclear. Further investigation is to needed to provide a better understanding of the mechanisms of these adverse reproductive outcomes, which in turn, may help to identify high risk subpopulations for clinical and public health intervention.

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